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STUDIES IN THE BIOCHEMISTRY OF CELLULOLYTIC MICROORGANISMS

I. CARBON BALANCES OF WOOD-ROTTING FUNGI IN SURFACE CULTURE[1]

BY D. R. WHITAKER[2]

Abstract

A carbon balance study was made of the metabolic products of 40 species of wood-rotting fungi, grown in surface culture on a glass wool support in a glucose–urea medium. Sixteen species were tested under both continuous and restricted aeration. The categories of product determined were: carbon in carbon dioxide evolved during incubation, carbon in mycelium, and carbon in the culture filtrate as total carbon, as residual glucose, as volatile acids, as volatile neutrals, and as carbon dioxide. The resulting carbon balances indicated that, under the conditions tested, carbon dioxide and mycelium were the only major end products of the majority of species examined.

Introduction

Among the microorganisms that metabolize cellulose, the Basidiomycete wood-rotting fungi form one of the largest groups. Certain species attack preferentially the cellulose and hemicellulose components of wood, leaving the lignin in a brown residue, and hence are termed brown rots; others attack the lignin component as well and are termed white rots. Considerable economic loss results from the destruction of trees and wood products by these organisms and a major interest in them concerns the preservation of wood against their attacks.

In recent years, there has been an increasing interest in their biochemistry. Especially extensive series of studies have been made by J. H. Birkinshaw and his co-workers on the identification of metabolic products of certain species, and by F. F. Nord and his co-workers on certain aspects of their enzymology. A recent review is that of Nord and Vitucci (4). To the best of the writer's knowledge, however, no general survey of the metabolic product pattern of the group has hitherto been made. Such a survey of 40 species representing 21 genera of wood-rotting fungi, grown in surface culture on a glucose medium, is the subject of this report.

The general design followed is patterned after, though not as extensive as, that of H. Raistrick and his co-workers in their classical study of the lower

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[2] *Biochemist, Industrial Utilization Investigations.*

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fungi (5). It consists essentially in determining, largely with the use of wet combustion, the partition of carbon between various classes of metabolic products resulting from the growth of surface cultures of fungi in a medium of known initial carbon content. The use of surface cultures of wood-rotting fungi has the disadvantage that growth is frequently very slow, usually much slower than in shake cultures, but for the purpose of an initial survey it was considered that surface cultures would give a truer picture of the biochemical potentials of the group.

Experimental

Cultural Conditions

Cultures were obtained from the collections of Dr. C. W. Fritz[1] and of Dr. Mildred K. Nobles[2]. Strain designations are those of the original collections, those of the first collection being characterized by the prefix A or S. The inoculum for each culture flask was the mycelium from a subculture on a malt agar slope in an 8 × 1 in. tube, incubated in the dark at 26° C. for 10 to 14 days. The mycelium was detached from the slope with a sterile knife after adding 20 ml. of water, and broken into fine fragments by shaking in a glass-stoppered tube with glass beads.

Each culture flask contained 800 ml. of medium and 1.5 gm. of glass wool. The medium was derived from Czapek-Dox solution by substituting urea for sodium nitrate as the nitrogen source, by substituting potassium sulphate for potassium chloride as the main potassium source, by reducing the ferrous sulphate concentration, and by adding certain vitamins and trace elements. Its composition is given in Table I. The presence of urea in this medium caused sterilization by autoclave to give rise to products inhibiting (or for some species preventing) the growth of a number of wood-rotting fungi.

TABLE I

Composition of medium per liter

Glucose	50 gm.
Urea	0.8 gm.
Magnesium sulphate heptahydrate	0.5 gm.
Potassium dihydrogen phosphate	1.0 gm.
Potassium sulphate	0.4 gm.
Ferrous sulphate heptahydrate	0.01 gm.
Thiamine hydrochloride	0.5 mgm.
Pyridoxine hydrochloride	0.1 mgm.
Biotin	0.005 mgm.
Boric acid	0.06 mgm.
Ammonium heptamolybdate tetrahydrate	0.04 mgm.
Cupric sulphate pentahydrate	0.2 mgm.
Zinc sulphate heptahydrate	2.0 mgm.
Manganese dichloride tetrahydrate	0.08 mgm.
Cobaltous chloride hexahydrate	0.4 mgm.
Calcium nitrate tetrahydrate	1.2 mgm.

[1] *Forest Products Laboratories of Canada, Dominion Forest Service, Ottawa, Canada.*

[2] *Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada.*

The urea was therefore sterilized separately by filtration of a solution through a Morton sintered-glass filter, with subsequent addition to the main solution sterilized by autoclave (15 min., 20 lb. pressure). The final pH of the medium was 4.6; 800 ml. of medium required 6.0 ml. of 1.0 *N* sodium hydroxide to bring it to phenolphthalein neutrality. The initial carbon content was taken as that of the glucose and urea in 800 ml. of medium, the former value being determined by analysis of the glucose concentrate added to each flask. The carbon content of the inoculum, being less than 1% of the total initial carbon, was ignored.

It was also found in initial trials of a number of species that the usual conditions of surface culture were unsatisfactory. Mycelial fragments, because of the absence of sporulation on laboratory media common in these organisms, had to be used as inocula; these tended to submerge and either did not develop further or developed very slowly. This tendency was overcome by adding sufficient glass wool to prevent submersion of the inoculum. Approximately 1.5 gm. samples of Pyrex glass wool were dried for 24 hr. at 110° C., cooled *in vacuo*, weighed, teased out, and added to the culture flasks before sterilization. For many species this support appeared essential for surface felt development and for others it permitted more rapid growth.

The culture flasks and accessory equipment are illustrated in Fig. 1. Inoculation and addition or removal of medium were made through the glass joint in the flask. Air entering the flask was freed of carbon dioxide, equilibrated with water, reduced in pressure, and filtered free of contaminants. Air leaving the flask was filtered and freed of volatile products and most of the water in a cold trap. Its last traces of water were absorbed in a Schwartz tube and its carbon dioxide absorbed in a weighed Nesbitt bulb packed with Anhydrone and 12-20 mesh Ascarite. The bubbler on the air inlet side of the flask indicated the approximate aeration rate and also indicated the presence of leaks if bubbling continued with the end Schwartz tube closed.

All species were aerated throughout the incubation period at a rate of about 50 ml. of air per minute. In addition, certain species were also tested with aeration restricted after the first 10 days to 30 min. per day at the same aeration rate, the air inlet and final outlet being closed during intervening periods. The incubation temperature was 26° C. and the flasks were kept in darkness except for brief daily examinations.

At five-day intervals, carbon dioxide production was measured by weighing the Nesbitt bulb, thereby providing an arbitrary index of the progress of metabolism. The object was to prevent, if possible, continuation of metabolism to the point where initially formed metabolic products were degraded as a result of exhaustion of glucose.

Analytical Methods

At the end of incubation, a malt agar slope was first inoculated from the mycelium of each flask for subsequent incubation and examination for contaminants. The medium and the contents of the cold trap were filtered

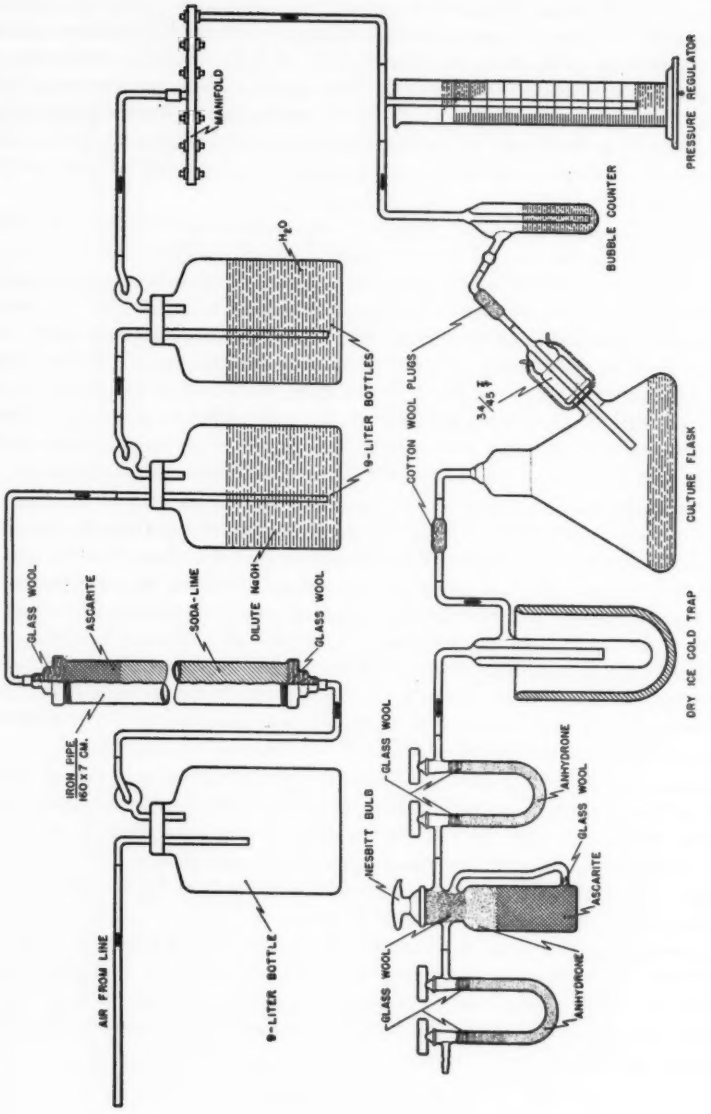
MANIFOLD
PRESSURE REGULATOR
BUBBLE COUNTER
H_2O
9-LITER BOTTLES
COTTON WOOL PLUGS
$3\frac{3}{45}$ ♀
CULTURE FLASK
DRY ICE COLD TRAP
GLASS WOOL
ASCARITE
SODA-LIME
DILUTE NaOH
GLASS WOOL
IRON PIPE
60 X 7 CM.
AIR FROM LINE
9-LITER BOTTLE
NESBITT BULB
GLASS WOOL
ANHYDRONE
GLASS WOOL
ASCARITE
GLASS WOOL
ANHYDRONE

FIG. 1.

through a tared sintered-glass funnel to which the mycelium, after mechanical separation from the glass wool, was added. Usually, this latter operation was extremely easy, the mycelium peeling off cleanly from the glass wool, but in the few cases when it was more difficult, only a portion of the mycelium was separated. The glass wool was added to a tared beaker and the flask, glass wool, and mycelium successively washed with carbon dioxide – free distilled water until the volume of filtrate was 1 liter. Samples of filtrate, pending analysis, were frozen in plastic tubes at −6.5° C. The tared beaker with the glass wool and the funnel with the mycelium were dried for 24 hr. at 90° C. and then for one week *in vacuo* over Anhydrone before weighing.

The carbon initially supplied in the medium should be accountable as the sum of three fractions: carbon evolved as carbon dioxide during incubation, carbon in solution in the culture filtrate, and carbon in mycelium. The carbon in these fractions was determined as follows:

Carbon in Carbon Dioxide

This was calculated from the measured increase in weight of the Nesbitt bulbs.

Carbon in Solution

Aliquots of culture filtrate were analyzed for total carbon by wet combustion by the method of Friedemann and Kendall (2) as modified by Wells *et al.* (9). The method consists essentially of chromic acid oxidation in a boiling sulphuric–phosphoric acid solution, with absorption in alkali and titration of the carbon dioxide evolved. In preliminary trials on a variety of test compounds, recoveries of 98-100% were obtained provided the volume of test solution did not exceed 10 ml. This accuracy was considered sufficient for the purpose in view.

Carbon in Mycelium

The dry weight of mycelium was obtained by subtracting the weight of the glass wool from the combined increase in weight of the tared filter and beaker. The bulk of the mycelium was detached from the filter, weighed, ground in a porcelain mortar, and reweighed to correct for moisture absorbed during grinding. The carbon content was then determined by wet combustion with correction of sample weights to original dry weight.

Carbon in solution in the culture filtrate was subdivided as follows:

a. *Carbon in Residual Glucose*

Residual glucose was determined by three different types of procedures:

1. By polarimeter,
2. By the alkaline oxidation method of Hinton and Macara (3),
3. By the copper reduction methods of Shaffer and Hartmann (6) or of Somogyi (7).

For comparison of the initial and final glucose concentrations, results are expressed as the concentrations for a volume of 800 ml. of culture filtrate. Carbon in residual glucose is calculated from the results of the copper reduction methods, which are considered to be the most specific. The other methods were used, as was done by Raistrick *et al.* (5), to test for the presence of optically active or readily oxidized metabolic products that might cause discrepancies between the three results.

b. *Carbon in Volatile Neutral Compounds*

One hundred milliliters of culture filtrate, neutralized to phenolphthalein with 0.1 *N* sodium hydroxide, was distilled over glass wool in a flask assembly wrapped in asbestos to minimize reflux. Distillate was collected at the rate of 3-5 ml. per minute to a total of 50 ml. An equal volume of carbon dioxide – free water was then added to the flask and a further 50 ml. of distillate collected. Carbon in the distillates was then determined by wet combustion.

c. *Carbon in Volatile Acids*

The procedure of Birkinshaw and Raistrick (1) was followed. The method consists essentially of repeated vacuum distillation of acidified culture filtrate, with trapping of volatile acids in baryta. The baryta solution is neutralized with sulphuric acid, acidified, boiled under reflux to remove carbon dioxide, neutralized, filtered to remove barium sulphate, and evaporated to dryness. The carbon in the resulting dry barium salts is then determined by wet combustion.

d. *Carbon Dioxide in Solution*

Carbon dioxide and its salts in solution were determined by the manometric method of Van Slyke (8).

e. *Carbon Unaccounted for*

Carbon listed in this category is the difference between total carbon in solution and the sum of carbon in the preceding categories. It includes therefore carbon in products not falling into these classes.

The above scheme gives no separate estimation of nonvolatile acids. As an approximate index of total acid production, the pH and titratable acidity of the diluted culture filtrate were determined. The pH was measured with a glass electrode. Titratable acidity was determined as the first step in the analysis for volatile neutral compounds and the results are expressed as the total change in titratable acidity: ml. 1.00 *N* sodium hydroxide required to neutralize 1000 ml. of the diluted culture filtrate less the amount required to neutralize 800 ml. of medium. As the uptake of phosphate by the organism can affect both pH and titratable acidity, the results are considered really significant indices of organic acid production only when the change is great.

Results and Discussion

The carbon balances of 40 species, including 16 with both continuous and restricted aeration, are listed in Table II. It is apparent from these balances

TABLE II A

CARBON BALANCES: *Collybia*, *Coniophora*, AND *Corticium*

	Species, catalogue No., and type of rot				
	Collybia velutipes Curt. ex Fr. S222 White		*Coniophora puteana* (Schum. ex Fr.) Karst S31 Brown		*Corticium vellereum* Ellis and Cr. 17657 Brown
	Incubation, days				
	32		33		28
	Aeration				
	Cont.	Rest.	Cont.	Rest.	Cont.
Carbon supplied, gm.	15.5	15.5	15.9	15.9	16.5
Final carbon distribution, gm.					
In CO_2	4.70	2.46	7.61	3.66	4.58
In solution	4.70	9.28	3.12	9.54	9.26
In mycelium	6.01	3.52	4.33	2.36	2.12
% accounted for	99.4	98.7	95.0	97.9	97.0
Carbon in solution, gm.					
As residual glucose	4.35	9.22	2.24	7.56	8.64
As volatile neutrals	0.19	0.40	0.25	1.29	0.17
As volatile acids	0.00	0.04	0.15	0.00	0.15
As CO_2	0.004	0.009	0.001	0.007	0.00
Unaccounted for	0.16	0.00	0.48	0.68	0.29
Glucose concentration, gm./100 ml.					
Initial	4.80	4.80	4.97	4.97	5.12
Final					
By copper reduction	1.36	2.88	0.70	2.36	2.70
By polarimeter	1.41	2.88	0.66	2.50	2.74
By iodine oxidation	1.34	2.90	0.80	2.63	2.85
Culture filtrate pH	4.4	5.6	2.0	2.4	4.3
Titratable acidity change, ml. 0.1 *N* acid	−4.60	−2.76	28.8	13.0	4.1
Mycelium					
Dry weight, gm.	13.59	8.06	9.76	5.20	4.89
% carbon	44.2	43.7	44.3	45.3	43.3

TABLE II B

CARBON BALANCES: *Daedalea, Coniophora* AND *Favolus*

	Species, catalogue No., and type of rot				
	Daedalea confragosa Bolt. ex Fr. S166 White		*Daedalea unicolor* (Bull.) Fr. 11265 White	*Echinodontium tinctorium* Ell. and Ev. S32 Brown	*Favolus europeus* Klotzsch S97 White
	Incubation, days				
	43		28	43	42
	Aeration				
	Cont.	Rest.	Cont.	Cont.	Cont.
Carbon supplied, gm.	16.2	16.2	16.5	16.6	16.6
Final carbon distribution, gm.					
In CO_2	6.67	1.30	7.13	0.65	0.51
In solution	4.81	12.78	6.76	15.08	15.27
In mycelium	4.12	1.58	1.95	0.62	0.36
% accounted for	96.3	97.6	95.8	98.8	97.0
Carbon in solution, gm.					
As residual glucose	4.19	11.26	4.99	14.63	14.68
As volatile neutrals	0.16	0.17	0.00	0.13	0.02
As volatile acids	0.00	0.00	0.01	0.01	0.03
As CO_2	0.003	0.009	0.00	0.007	0.001
Unaccounted for	0.46	1.34	1.77	0.30	0.54
Glucose concentration, gm./100 ml.					
Initial	5.01	5.01	5.12	5.15	5.15
Final					
By copper reduction	1.31	3.52	1.56	4.57	4.59
By polarimeter	1.30	3.56	1.56	4.55	4.62
By iodine oxidation	1.38	3.52	1.72	4.57	4.56
Culture filtrate					
pH	4.1	4.9	3.8	6.2	5.9
Titratable acidity change, ml. 0.1 *N* acid	2.19	1.47	4.37	−0.45	3.8
Mycelium					
Dry weight, gm.	8.81	3.67	4.49	1.43	0.81
% carbon	46.8	42.9	43.4	43.3	44.2

TABLE II C

CARBON BALANCES: *Fomes* AND *Hericeum*

	Species, catalogue No., and type of rot					
	Fomes fomentarius (L. ex Fr.) Kickx S213 White		*Fomes igniarius* (L. ex Fr.) Gill S237 White	*Fomes pinicola* (Sw.) Cooke S45 Brown	*Fomes roseus* (Alb. and Schw. ex Fr.) Cooke 17572 Brown	*Hericeum erinaceus* (Fr.) Pers. F2167 White
	Incubation, days					
	37		45	42	44	44
	Aeration					
	Cont.	Rest.	Cont.	Cont.	Cont.	Cont.
Carbon supplied, gm.	15.9	15.9	15.8	16.6	16.3	16.3
Final carbon distribution, gm.						
In CO_2	1.94	0.82	2.49	0.59	1.56	2.04
In solution	12.18	14.41	10.44	15.21	13.40	13.25
In mycelium	1.35	0.74	2.45	0.43	0.49	0.95
% accounted for	97.4	100.5	97.5	97.6	95.1	99.4
Carbon in solution, gm.						
As residual glucose	12.16	13.95	10.53	13.99	13.44	12.80
As volatile neutrals	0.03	0.04	0.07	0.13	0.00	0.00
As volatile acids	0.10	0.09	0.01	0.42	0.00	0.01
As CO_2	0.002	0.002	0.001	0.00	0.002	0.002
Unaccounted for	0.00	0.33	0.00	0.67	0.00	0.44
Glucose concentration, gm./100 ml.						
Initial	4.97	4.97	4.90	5.15	5.00	5.00
Final						
By copper reduction	3.80	4.36	3.29	4.37	4.20	4.00
By polarimeter	3.76	4.33	3.26	4.37	4.17	4.01
By iodine oxidation	3.80	4.36	3.26	4.33	4.22	4.01
Culture filtrate						
pH	4.6	5.1	3.5	3.1	4.5	4.7
Titratable acidity change, ml. 0.1 *N* acid	3.66	2.22	5.4	4.32	2.40	3.75
Mycelium						
Dry weight, gm.	3.05	1.83	5.41	1.02	1.19	2.17
% carbon	44.3	40.7	45.3	42.5	41.3	43.6

TABLE II D

CARBON BALANCES: *Hydnum*, *Lentinus*, *Lenzites*, AND *Merulius*

	Species, catalogue No., and type of rot					
	Hydnum septentrionale Fr. S91 White	*Lentinus lepideus* Fr. S62 Brown		*Lenzites trabea* Pers. ex Fr. A92 Brown		*Merulius brassicaefolius* Schw. 21306 Brown
	Incubation, days					
	43	43		32	32	35
	Aeration					
	Rest.	Cont.	Rest.	Cont.	Rest.	Cont.
Carbon supplied, gm.	16.2	16.2	16.2	15.5	15.5	16.5
Final carbon distribution, gm.						
In CO_2	2.94	1.64	1.42	7.57	2.77	4.17
In solution	11.10	13.28	13.65	4.85	11.90	10.21
In mycelium	1.29	1.10	0.83	2.54	1.11	1.73
% accounted for	94.4	98.8	98.2	96.7	102	97.6
Carbon in solution, gm.						
As residual glucose	9.16	12.96	12.38	3.68	10.30	9.80
As volatile neutrals	1.73	0.13	0.27	0.13	0.51	0.07
As volatile acids	0.22	0.08	0.02	0.00	0.11	0.00
As CO_2	0.001	0.002	0.009	0.001	0.008	0.001
Unaccounted for	0.00	0.11	0.97	1.04	0.98	0.34
Glucose concentration, gm./100 ml.						
Initial	5.01	5.01	5.01	4.80	4.80	5.12
Final						
By copper reduction	2.86	4.05	3.87	1.15	3.22	3.06
By polarimeter	2.81	4.07	3.92	1.10	3.12	3.09
By iodine oxidation	2.88	4.17	4.27	1.56	3.62	3.18
Culture filtrate						
pH	4.9	4.4	4.7	3.2	4.2	2.3
Titratable acidity change, ml. 0.1 *N* acid	3.67	2.42	3.06	8.8	4.8	16.7
Mycelium						
Dry weight, gm.	3.03	2.52	1.69	5.34	2.40	3.82
% carbon	42.7	43.5	48.9	47.5	46.1	45.2

TABLE II E

CARBON BALANCES: *Peniophora* AND *Pholiota*

	Species, catalogue No., and type of rot					
	Peniophora gigantea (Fr.) S73 Brown		*Peniophora mutata* (Peck) Höhn and Litsch 17558 White	*Pholiota adiposa* Fr. S30 Brown		*Pholiota spectabilis* Fr. 8459 Brown
	Incubation, days					
	43		28	39		44
	Aeration					
	Cont.	Rest.	Cont.	Cont.	Rest.	Cont.
Carbon supplied, gm.	16.2	16.2	16.5	15.9	15.9	16.3
Final carbon distribution, gm.						
In CO_2	5.16	4.87	5.73	0.53	0.54	3.21
In solution	9.17	9.62	8.48	14.59	14.26	10.83
In mycelium	1.07	0.92	1.97	0.60	0.64	1.48
% accounted for	95.1	95.1	98.2	98.8	96.8	95.1
Carbon in solution, gm.						
As residual glucose	4.64	4.42	8.36	14.40	13.60	10.20
As volatile neutrals	3.74	4.47	0.00	0.07	0.00	0.00
As volatile acids	0.00	0.22	0.01	0.00	0.00	0.03
As CO_2	0.001	0.001	0.00	0.001	0.002	0.003
Unaccounted for	0.79	0.51	0.11	0.12	0.66	0.60
Glucose concentration, gm./100 ml.						
Initial	5.01	5.01	5.12	4.93	4.93	5.00
Final						
By copper reduction	1.45	1.38	2.61	4.50	4.25	3.19
By polarimeter	1.40	1.34	2.64	4.48	4.21	3.24
By iodine oxidation	1.43	1.38	2.74	4.60	4.20	3.20
Culture filtrate						
pH	5.5	5.7	4.6	4.7	5.7	3.7
Titratable acidity change, ml. 0.1 *N* acid	0.73	0.97	−0.97	1.42	2.14	7.49
Mycelium						
Dry weight, gm.	2.68	2.08	4.55	1.46	1.51	3.32
% carbon	40.0	44.1	43.2	40.9	42.7	44.7

TABLE II F

CARBON BALANCES: *Pleurotus* AND *Polyporus*

	Species, catalogue No., and type of rot					
	Pleurotus ostreatus Jacq. ex Fr. S9 White	*Polyporus abietinus* Dicks. ex Fr. A18 White		*Polyporus balsameus* Peck. A14 Brown	*Polyporus borealis* Fr. S25 White	
	Incubation, days					
	35	37		43	45	
	Aeration					
	Cont.	Cont.	Rest.	Cont.	Cont.	Rest.
Carbon supplied, gm.	16.5	15.9	15.9	16.6	15.8	15.8
Final carbon distribution, gm.						
In CO_2	1.82	4.60	3.02	4.27	2.26	1.27
In solution	11.18	7.59	10.58	9.59	11.50	13.15
In mycelium	2.78	2.86	1.96	2.14	1.80	0.96
% accounted for	95.7	95.0	97.9	96.4	98.7	97.5
Carbon in solution, gm.						
As residual glucose	11.17	4.76	9.48	8.42	11.67	13.12
As volatile neutrals	0.00	0.13	0.07	0.17	0.04	0.18
As volatile acids	0.00	0.36	0.54	0.06	0.00	0.00
As CO_2	0.002	0.002	0.001	0.00	0.001	0.002
Unaccounted for	0.01	2.34	0.49	0.84	0.00	0.00
Glucose concentration, gm./100 ml.						
Initial	5.12	4.97	4.97	5.15	4.90	4.90
Final						
By copper reduction	3.49	1.49	2.96	2.63	3.65	4.10
By polarimeter	3.51	1.40	2.97	2.61	3.70	4.15
By iodine oxidation	3.60	1.45	3.02	2.70	3.73	4.10
Culture filtrate						
pH	4.5	3.5	3.6	3.6	4.5	5.5
Titratable acidity change, ml. 0.1 *N* acid	−1.35	0.82	2.26	7.89	4.2	2.3
Mycelium						
Dry weight, gm.	6.72	6.42	4.46	4.48	4.01	2.21
% carbon	41.4	44.6	44.0	47.7	45.0	43.6

TABLE II G

CARBON BALANCE: *Polyporus*

	Species, catalogue No., and type of rot				
	Polyporus dryophilus Berk. S185 White	*Polyporus fibrillosus* Karst S106 Brown	*Polyporus mollis* Pers. ex Fr. S116 Brown	*Polyporus pargamenus* Fr. A43 White	
	Incubation, days				
	42	35	44	32	
	Aeration				
	Cont.	Cont.	Cont.	Cont.	Rest.
Carbon supplied, gm.	16.6	16.5	16.3	15.5	15.5
Final carbon distribution, gm.					
In CO_2	2.26	3.72	3.73	6.32	2.94
In solution	12.35	10.04	11.50	6.86	11.10
In mycelium	2.00	2.07	0.78	2.01	1.36
% accounted for	100	95.7	98.2	98.2	99.4
Carbon in solution, gm.					
As residual glucose	11.88	8.16	11.52	6.24	11.07
As volatile neutrals	0.10	1.74	0.00	0.26	0.28
As volatile acids	0.08	0.07	0.01	0.00	0.01
As CO_2	0.00	0.003	0.00	0.004	0.001
Unaccounted for	0.29	0.07	0.00	0.36	0.00
Glucose concentration, gm./100 ml.					
Initial	5.15	5.12	5.00	4.80	4.80
Final					
By copper reduction	3.71	2.55	3.60	1.95	3.46
By polarimeter	3.66	2.50	3.64	1.90	3.44
By iodine oxidation	3.71	2.61	3.62	1.93	3.46
Culture filtrate					
pH	4.4	4.4	4.3	4.9	6.1
Titratable acidity change, ml. 0.1 *N* acid	1.02	0.65	2.50	−0.08	−0.04
Mycelium					
Dry weight, gm.	4.43	4.55	1.71	4.51	3.03
% carbon	45.1	45.5	45.5	44.6	44.8

TABLE II H

CARBON BALANCES: *Polyporus* AND *Poria*

	Species, catalogue No., and type of rot					
	Polyporus versicolor L. ex Fr. 21150 White	*Poria albipellucida* Baxter 16125 White	*Poria monticola* Murr. A121 Brown		*Poria subacida* (Peck.) Sacc. 17780 White	*Poria xantha* (Fr.) Cooke A74 Brown
	Incubation, days					
	28	35	60		35	39
	Aeration					
	Cont.	Cont.	Cont.	Rest.	Cont.	Cont.
Carbon supplied, gm.	16.5	16.5	15.8	15.8	16.5	15.9
Final carbon distribution, gm.						
In CO_2	6.07	2.78	0.59	0.57	1.00	2.03
In solution	3.23	10.98	14.49	14.50	14.21	12.00
In mycelium	6.74	2.07	0.33	0.14	0.79	1.31
% accounted for	97.0	95.8	97.4	96.2	97.0	96.3
Carbon in solution, gm.						
As residual glucose	2.72	10.08	13.72	14.62	14.08	11.20
As volatile neutrals	0.07	0.15	0.13	0.14	0.02	0.05
As volatile acids	0.01	0.02	0.36	0.07	0.05	0.00
As CO_2	0.001	0.00	0.00	0.002	0.003	0.00
Unaccounted for	0.43	0.73	0.28	0.00	0.08	0.75
Glucose concentration, gm./100 ml.						
Initial	5.12	5.12	4.90	4.90	5.12	4.93
Final						
By copper reduction	0.85	3.15	4.29	4.57	4.40	3.50
By polarimeter	0.80	3.16	4.30	4.60	4.40	3.52
By iodine oxidation	0.95	3.18	4.45	4.69	4.50	3.52
Culture filtrate						
pH	3.7	3.9	2.8	2.9	4.1	1.9
Titratable acidity change, ml. 0.1 *N* acid	0.22	1.50	9.1	8.3	5.45	50.0
Mycelium						
Dry weight, gm.	15.64	4.55	0.76	0.28	1.98	3.13
% carbon	43.1	45.5	44.2	49.2	40.1	42.0

TABLE II I

CARBON BALANCES: *Schizophyllum*, *Stereum*, AND *Trametes*

	Species, catalogue No., and type of rot				
	Schizophyllum commune Fr. A23 White	*Stereum sanguinolentum* Alb. and Schw. ex Fr. S67 Brown		*Trametes americana* Overholts A109 Brown	
	Incubation, days				
	22	39		45	
	Aeration				
	Cont.	Cont.	Rest.	Cont.	Rest.
Carbon supplied, gm.	16.6	15.9	15.9	15.8	15.8
Final carbon distribution, gm.					
In CO_2	8.52	1.79	1.56	5.59	3.37
In solution	5.06	12.96	13.01	6.12	11.28
In mycelium	2.51	0.79	0.62	3.77	0.86
% accounted for	97.6	97.5	95.6	98.2	98.2
Carbon in solution, gm.					
As residual glucose	2.05	12.76	11.56	5.92	10.59
As volatile neutrals	1.52	0.10	1.14	0.23	0.28
As volatile acids	0.07	0.01	0.00	0.00	0.00
As CO_2	0.002	0.002	0.005	0.00	0.001
Unaccounted for	1.42	0.09	0.31	0.00	0.41
Glucose concentration, gm./100 ml.					
Initial	5.15	4.93	4.93	4.90	4.90
Final					
By copper reduction	0.64	3.99	3.61	1.85	3.31
By polarimeter	0.63	4.04	3.69	1.89	3.32
By iodine oxidation	0.99	4.00	3.65	1.90	3.35
Culture filtrate					
pH	4.5	5.7	5.6	3.6	4.0
Titratable acidity change, ml. 0.1 *N* acid	6.20	2.21	4.73	6.9	12.0
Mycelium					
Dry weight, gm.	5.78	1.86	1.45	8.20	1.94
% carbon	43.4	42.5	42.6	46.0	44.2

TABLE II J

CARBON BALANCES: *Trametes*, *Trechispora*, AND *Volvaria*

	Species, catalogue No., and type of rot					
	Trametes serialis Fr. S150 Brown		*Trametes suaveolens* (L.) Fr. S171 White		*Trechispora raduloides* (Karst) Reg. 17146 Brown	*Volvaria bombycina* Peck 21190 Brown
	Incubation, days					
	33		32		35	44
	Aeration					
	Cont.	Rest.	Cont.	Rest.	Cont.	Cont.
Carbon supplied, gm.	15.9	15.9	15.5	15.5	16.5	16.3
Final carbon distribution, gm.						
In CO_2	7.43	3.08	5.23	2.72	5.49	4.76
In solution	4.26	10.96	8.29	11.10	8.60	8.16
In mycelium	3.52	1.45	2.02	1.61	1.70	2.56
% accounted for	95.7	97.5	100	99.4	95.7	95.1
Carbon in solution, gm.						
As residual glucose	3.68	9.66	7.87	11.05	5.88	7.24
As volatile neutrals	0.18	0.69	0.23	0.24	1.74	0.00
As volatile acids	0.16	0.10	0.03	0.03	0.07	0.21
As CO_2	0.002	0.001	0.004	0.008	0.00	0.00
Unaccounted for	0.23	0.51	0.16	0.00	0.91	0.71
Glucose concentration, gm./100 ml.						
Initial	4.87	4.87	4.80	4.80	5.12	5.00
Final						
By copper reduction	1.15	3.02	2.46	3.45	1.84	2.26
By polarimeter	1.13	2.97	2.52	3.48	1.80	2.23
By iodine oxidation	1.24	3.02	2.57	3.50	1.94	2.40
Culture filtrate						
pH	2.9	3.8	4.3	4.8	5.6	4.6
Titratable acidity change, ml. 0.1 *N* acid	0.34	2.49	−0.88	−0.40	2.62	1.48
Mycelium						
Dry weight, gm.	8.20	3.19	4.58	3.66	3.56	5.91
% carbon	42.9	45.4	44.2	44.1	47.7	43.4

that, under the conditions tested, carbon dioxide and mycelium were generally the only major end products of the species examined. As an approximate measure of the extent to which metabolism is channelled toward carbon dioxide and mycelium production, the ratio:

$$\frac{\text{carbon in carbon dioxide and mycelium}}{\text{total carbon accounted for } \textit{minus} \text{ carbon in residual glucose}}$$

was used as an index: the values of this index for the 39 species under continuous aeration were distributed as follows: 0.9-1.0 (22 species), 0.8-0.9 (eight species), 0.7-0.8 (four species), 0.6-0.7 (two species), 0.5-0.6 (three species). The values thus tend to be very high.

Restriction of aeration tended, as might be expected, to lower the index. However, the present data, particularly when the difference between the denominator terms is small, are not considered sufficiently exact to illustrate more than a trend. Of the 16 species tested under both continuous and restricted aeration, the index of the restricted member was lower for nine species (by from 0.20-0.35 for five species, from 0.10-0.20 for four), was little changed for six species (change $\pm$ 0.1), and was higher for one species (by 0.4). At the same time whenever growth (and presumably therefore oxygen demand) was heavy, restriction of aeration slowed up the rate of glucose utilization.

Some of the implications of these results will be further discussed in Part II of this series.

Acknowledgment

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STUDIES IN THE BIOCHEMISTRY OF CELLULOLYTIC MICROORGANISMS

II. METABOLIC PRODUCTS OF *POLYPORUS ABIETINUS, PENIOPHORA GIGANTEA,* AND *HYDNUM SEPTENTRIONALE*[1]

By D. R. Whitaker[2] and Phyllis E. George[3]

Abstract

The metabolic product causing a high "carbon unaccounted for" fraction in the carbon balance of *Polyporus abietinus* Dicks. ex Fr., was found to be D-mannitol. The metabolic product causing a high volatile neutral fraction in the carbon balances of *Peniophora gigantea* (Fr.) Massee and *Hydnum septentrionale* Fr. was found to be ethanol.

Introduction

In Part I of this series, the results of a carbon balance survey of 40 species of wood-rotting fungi were reported (8). Although the carbon balances of most species indicated that carbon dioxide and mycelium were the only major products formed in surface culture on the glucose medium used, those of a few species appeared of sufficient interest to warrant further investigation.

The carbon balance of *Polyporus abietinus* Dicks. ex Fr. A18 was of interest for the unusually high figure of "carbon unaccounted for" in solution, i.e. carbon in the culture filtrate not accounted for as residual glucose, volatile neutrals, volatile acids, and carbon dioxide and its salts. After 37 days' incubation on a medium containing 15.9 gm. of carbon initially, this amounted to 2.34 gm. out of a total of 7.59 gm. of carbon in solution. The change in titratable acidity was small and estimations of residual glucose by copper reduction, by polarimeter, and by alkaline iodine oxidation were in approximate agreement, suggesting that the "carbon unaccounted for" fraction, unless very heterogeneous, was not acidic, not appreciably optically active, and not easily oxidized.

The carbon balances of *Peniophora gigantea* (Fr.) Massee S73 and of *Hydnum septentrionale* Fr. S91 were of interest because of the comparatively high content of carbon in the volatile neutral fraction. For *Peniophora gigantea* under continuous aeration, this fraction contained 3.74 gm. of

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carbon out of 16.2 gm. supplied; for *Hydnum septentrionale*, under restricted aeration, this fraction contained 1.73 gm. out of 16.2 gm. supplied.

The nature of the metabolic products in these fractions is the subject of this report.

Experimental and Results

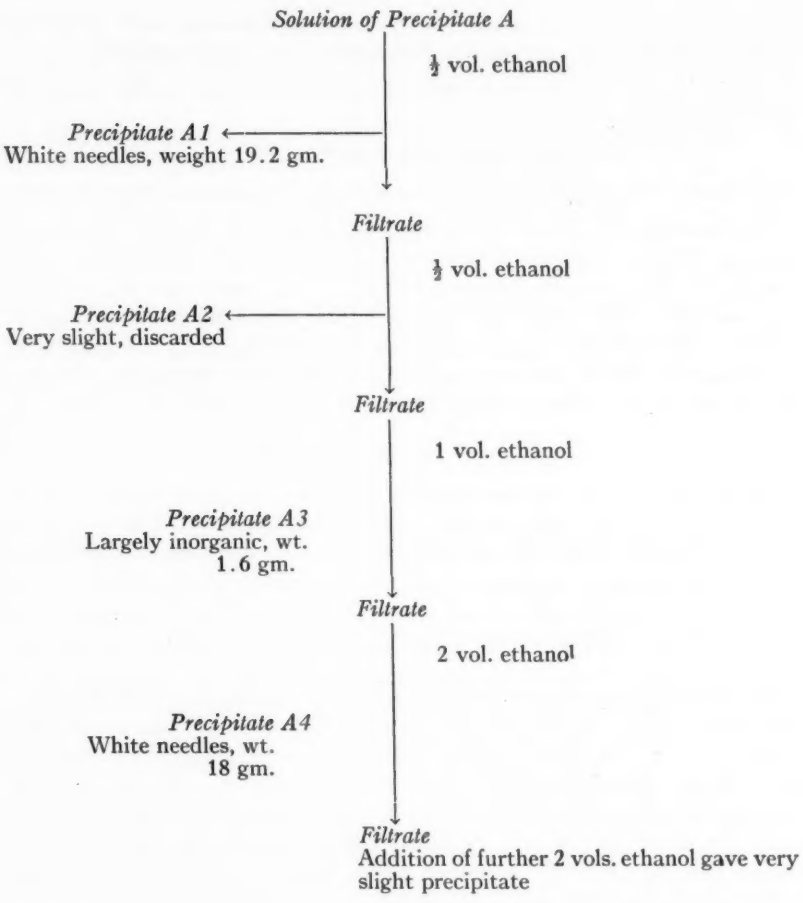
The "Carbon Unaccounted for" Fraction of Polyporus abietinus Dicks. ex Fries

To determine the nature of this fraction, 5 liters of medium, distributed over six 2500 ml. Fernback flasks each containing 1.5 gm. of glass wool, were inoculated with mycelial suspensions from 13-day malt agar slopes of *P. abietinus* A18. The flasks were plugged with cotton wool, the manner of aeration differing in this respect from that previously applied. After 49 days' incubation at 25° C., the contents of the flasks were filtered, the culture filtrate having the following properties:

Volume	4270 ml.
pH	3.9
Residual glucose (by polarimeter)	0.22 gm. per 100 ml.
Total glucose carbon	3.8 gm.
Total carbon (wet combustion)	20.9 gm.

The culture filtrate was concentrated by vacuum distillation (bath temperature below 45° C.) to 920 ml. and the concentrate was cleared in the cold (about 0° C.) at pH 7 with a slight excess of saturated lead acetate solution, the sediment centrifuged down being largely inorganic. Excess lead was removed by centrifuging after addition of 2 *N* sulphuric acid to slight excess. After adding, at pH 3.5, two volumes of ethanol and leaving the solution to stand overnight at 30° F., a further heavy, largely inorganic fraction was centrifuged down. The resulting solution was then concentrated by vacuum distillation (bath temperature below 35° C.) to 110 ml. and four volumes of ethanol was added. After standing overnight at 30° F., a heavy precipitate (Precipitate A) came down and was filtered off. Addition to the filtrate of a further three volumes of ethanol caused only a very slight precipitation.

After it was washed with cold 80% (v/v) ethanol and dried *in vacuo*, Precipitate A weighed 47 gm. and gave a cloudy melt, with browning, over the range 162-167° C. It was dissolved in 250 ml. of water and fractionated with ethanol. After each ethanol addition, the solution was allowed to stand at 0° C., and the resulting precipitate was washed with cold ethanol of the same concentration as the solution from which it had precipitated; the washings were added to the main filtrate. The procedure was as follows:



Precipitates A1 and A4 were shown to be D-mannitol by evidence summarized in Table I. Melting points are uncorrected. Mixed melting points are with Difco D-mannitol and with derivatives prepared from it. The acetyl derivatives were prepared by the procedure of Patterson and Todd (5), using acetic anhydride and fused zinc chloride as acetylating agent, with one recrystallization from aqueous methanol. The benzal derivatives were prepared by the method of Pette (6), using benzaldehyde and phosphorus pentoxide, and were recrystallized twice from chloroform – petroleum ether. For both these derivatives, a considerable range of melting points is reported in the literature. Those recorded in Table I are the highest noted. The literature value given for the specific rotation of D-mannitol in 6% borax is

TABLE I

IDENTIFICATION OF PRECIPITATES A1 AND A4

	A1	A4	D-mannitol	Literature values
M.p. Mixed m.p.	165-66° C. 165-66° C.	166-67° C. 166-67° C.	166-67° C.	166° C. (2)
M.p., acetyl derivative Mixed m.p.	123-24° C. 123-24° C.	123-24° C. 123-24° C.	123-24° C.	126° C.* (5)
M.p., benzal derivative Mixed m.p.	211-12° C. 212-13° C.	211-12° C. 212-13° C.	212-13° C.	223-24° C.**(6)
$[\alpha]_D^{25}$ in water (c = 4.0%)	− 0.2° C.	+ 0.2° C.	− 0.1° C.	− 0.21° C. (2)
$[\alpha]_D^{20}$ in 6% borax solution (c = 4.0%)	+24.6° C.	+25.1° C.	+24.5° C.	+24.9° C. (7)

* *Hexa-acetyl-D-mannitol.*
** *Tribenzal-D-mannitol.*

calculated from the data of Raistrick and Young (7) for comparable concentrations, using the same factor as for quartz to convert readings from the mercury green to the sodium D line.

The 37.2 gm. of D-mannitol in precipitates A1 and A4 thus account for 14.7 gm. out of the 17.1 gm. difference between total carbon and residual glucose carbon in the original culture filtrate.

The Volatile Neutral Fractions of Peniophora gigantea and Hydnum septentrionale

Three Fernbach flasks, each containing 800 ml. of medium and 1.5 gm. of glass wool, were inoculated with mycelial suspensions of each organism. As before, the flasks were plugged with cotton. After 36 days' incubation at 26° C., the mycelia and glass wool were filtered off, the two culture filtrates neutralized and, by the addition of 120 gm. of sodium sulphate, partially saturated with salt. They were then distilled over glass wool with minimum reflux. The distillates were collected in 250 ml. fractions and, after collection of the third fraction, 750 ml. of water was added to the flask and a further three fractions collected.

For both organisms, the fourth and subsequent fractions had a negligibly small carbon content as determined by wet combustion and were not examined further. Both sets of the remaining fractions were optically inactive and showed no consumption of alkali on reflux with ethanolic potassium hydroxide. All gave a slight precipitate or cloudiness with Brady's reagent (2,4-dinitrophenylhydrazine in hydrochloric acid), indicating the presence of traces of a volatile carbonyl compound, and all gave positive iodoform reactions.

The major component of the volatile neutral fractions of both species was shown to be ethanol by the following evidence. On treatment of the first fractions with 3,5-dinitrobenzoyl chloride by the procedure of Lipscomb and Baker (4), derivatives were obtained of m.p. 91-92° C., undepressed on mixture with ethyl 3,5-dinitrobenzoate of m.p. 92-92.5° C. On treatment with *p*-nitrobenzoyl chloride by the procedure of Henstock (3), derivatives were obtained of m.p. 53-54° C., undepressed on mixture with ethyl *p*-nitrobenzoate of m.p. 53-54° C. Ethanol was estimated in the two sets of fractions by three methods, each differing in principle:

1. By dichromate oxidation of ethanol to acetic acid, with analysis of the dichromate consumed;

2. From the specific gravity at 25° C.;

3. By determining total carbon by wet combustion and expressing total carbon as ethanol.

Individually, none of these methods is specific for ethanol but when collectively their results are in approximate agreement, particularly those of Methods 1 and 3, they provide both an approximate quantitative estimate and a further confirmation of identification. These results are summarized in Table II. From the degree of agreement obtained, it is concluded that the estimates by the most sensitive method — dichromate oxidation — are approximately correct.

TABLE II

VOLATILE NEUTRAL FRACTIONS: *Peniophora gigantea* AND *Hydnum septentrionale*

Fraction	Ethanol concentration, gm./100 ml.		
	By dichromate oxidation	By specific gravity	By wet combustion
Peniophora gigantea			
1st	9.66	10.0	9.4
2nd	0.91		0.90
3rd	0.029		0.03
Hydnum septentrionale			
1st	2.41	3	2.2
2nd	0.20		0.22
3rd	0.021		0.02

Discussion

It is noteworthy that both metabolic products — mannitol and ethanol — are reduction products of glucose metabolism and hence are products whose formation would be expected to be suppressed under those cultural conditions in which growth of wood-rotting fungi is reasonably rapid, namely in highly aerated shake or submerged cultures. Suppression of ethanol formation by

aeration has been known since the work of Pasteur. The effect is illustrated in the preceding carbon balances of *Peniophora gigantea* S73. A similar need for restriction of aeration in the conversion of glucose to mannitol by certain aspergilli was found by Raistrick and his co-workers (1). As shown by its carbon balances (8), *Polyporus abietinus* A18 is not as sensitive as these aspergilli in this respect, possibly owing to its formation in surface culture of a firm, continuous pellicle whose thickness increases with aeration — although under none of the cultural conditions tested could the aeration rate be considered high.

In the preceding carbon balance survey, the typical metabolic end product pattern of surface cultures of wood-rotting fungi was found to be strongly biased towards mycelium and carbon dioxide formation. The three most marked exceptions to this typical pattern would be expected to conform to it under conditions permitting rapid growth. In general then, it is considered that the results of this survey suggest that in applications in which rapidity of growth is essential, the only potentially valuable bulk metabolic product of wood-rotting fungi is their mycelium.

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STUDIES OF VIRUS DISEASES OF STRAWBERRIES IN BRITISH COLUMBIA

I. THE REACTION OF THE BRITISH SOVEREIGN VARIETY AND THE INDICATOR *FRAGARIA VESCA* TO YELLOWS[1]

By Randal E. Fitzpatrick and Frances C. Mellor[2]

Abstract

British Sovereign, the commercial strawberry variety of British Columbia, has shown no field evidence of degeneration from the virus disease, yellows, that is attacking the Marshall variety in the Pacific Northwest. However, experimental inoculation of British Sovereign by stolon grafting to a yellows-infected Marshall plant proved the British Sovereign to be susceptible to yellows. As with the Marshall, infected plants were reduced in size and the foliage tended to flatten towards the ground, but, unlike the Marshall, there was no pronounced yellowing. When *Fragaria vesca* was grafted to a yellows-infected Marshall plant the reaction was rapid and severe. The older leaves flattened to the ground, the young runner tips hooked back, the newly developing leaves, though relatively well proportioned, were minute and yellow at the margins, and the plants eventually died.

Introduction

Etiology of Virus Diseases of Strawberry

The virus diseases of strawberry have for the most part been worked on independently by individuals and groups in the particular localities where the diseases have appeared. This, together with the fact that different varieties have been involved, precludes any but a wholly tentative summary of their etiology at this time. The following is a resumé of the various strawberry diseases reputed to be of virus origin.

Yellows (*Xanthosis*) and *Yellow-edge*

Yellows (xanthosis) was first described by Horne (7) in California in 1922. Plakidas (10) first proved the virus nature of the disease when he succeeded in obtaining transmission with the strawberry leaf aphid, *Capitophorus fragaefolii*, by taking insects from diseased plants and allowing them to feed for two weeks on healthy Marshall plants.

Yellow-edge was the name given by Harris (3) to what appears to be the same disease on the Royal Sovereign variety in England. Harris (2) obtained transmission by grafting together stolons of diseased and healthy plants. Later Harris and King (5), Harris and Hildebrand (4), and Hildebrand (6), using the same technique, showed that the wild strawberry species, *Fragaria vesca* and *F. virginiana*, were particularly susceptible to the strawberry viruses and hence were useful indicator plants. Subsequently Massee (8) obtained

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transmission of yellow-edge by colonizing healthy plants with aphids taken from diseased ones. More recently Prentice and Harris (14) and Prentice (12, 13) demonstrated that yellow-edge is the result of multiple infection by two or more different viruses which could be separated by varying the acquisition and transfer feeding periods of the vectors.

Crinkle

Crinkle was first described by Zeller and Vaughan (20) in Oregon on the Marshall variety. Zeller (18) obtained transmission by moving aphid colonies from diseased to healthy plants. Vaughan (15) found that, when aphids from crinkle-infected plants were transferred to healthy ones and allowed to feed on these for a week, after the first "shock" of the virus some of the plants seemed to recover. Massee (9), working with mild crinkle in England, obtained transmission by moving aphids from diseased to healthy plants and allowing them to feed for a month. In another experiment, using winged aphids, he transmitted severe crinkle in a similar manner. More recently Wood and Whitehead (16) presented evidence to show that plants suffering from severe crinkle are infected with two viruses, one persistent in the aphid and the other not, and that, when the acquisition feeding period is short (up to four hours), only the nonpersistent virus is transmitted.

Witches'-Broom

Witches'-broom was first described by Zeller (17) in Oregon. Transmission was obtained by feeding aphids for eight days on diseased plants and then colonizing them on healthy ones.

Stunt

Stunt was described by Zeller (19) in 1941 in Oregon. He stated that the disease was readily transmitted by the strawberry leaf aphid, but gave no details of the experimental work.

Leaf Roll

Berkeley and Plakidas (1), working in Ontario and New York respectively, showed a leaf roll condition to be graft transmissible. No insect transmission was attempted.

Dwarf

This disease, which was at one time considered to be caused by a virus (11), has since been shown to be due to nematode infestation.

The Virus Problem in British Columbia

During the last few years the Marshall strawberry in the Pacific Northwest has been rapidly deteriorating from the yellows disease. This has resulted in a situation of particular interest to the strawberry industry of British Columbia. In British Columbia, the main strawberry growing district is in the lower Fraser Valley. This district is immediately adjacent to a similar

area in the State of Washington, U.S.A. Climatically the areas are very similar, and it might be supposed that the same varieties and methods of culture would be adopted in both. This, however, is not the case. In Washington the Marshall strawberry is grown almost exclusively, whereas in British Columbia a local variety, the British Sovereign,* is favored.

The Marshall is an old variety, having been under cultivation since 1890. It has been grown on the Pacific coast for decades, often under other names, such as Oregon, Improved Oregon, Oregon Plum, Admiral Dewey, Banner, Rose, and Tuttle. The Marshall is usually grown in matted rows, and this method of culture is used almost exclusively in Washington. It is susceptible to both yellows and crinkle, and to witches'-broom and stunt. Indeed, it was on the Marshall that all these diseases were first described. Yellows and crinkle have been present in Washington for years, but it is only comparatively recently that serious degeneration has occurred.

The British Sovereign on the other hand is a more recent variety than the Marshall. It originated as a chance seedling in the Fraser Valley, where it has gained such popularity that it is now grown to the virtual exclusion of all others. The British Sovereign does best under the individual plant or "hill" system of culture, and this is the method used in British Columbia. The existing stocks of British Sovereign are free of visible virus disease and there is no evidence of degeneration in them.

The contrast in the disease situation of the two varieties is naturally arousing considerable interest both in Washington and in British Columbia. The Washington growers for their part are considering the British Sovereign as a possible replacement for the Marshall; the British Columbia growers, conscious of the experience in Washington with the Marshall, are becoming alarmed lest a similar degeneration overtake the British Sovereign.

The most obvious questions to be answered were: first, is the British Sovereign tolerant of the yellows disease of Marshall, or has the stock in the Fraser Valley merely escaped serious contamination; and second, would *Fragaria vesca* prove as satisfactory an indicator plant for the yellows of the Pacific Northwest as it has proved for analogous diseases in other parts of the world? The experiments described in this paper were designed to answer these two questions.

Experimental

Material and Method

The original source of inoculum was a Marshall strawberry plant showing typical symptoms of severe yellows. The British Sovereign plants were from certified stock which appeared healthy and vigorous. Unfortunately it was not possible at that time to determine whether they themselves were carrying any latent virus, but this point will be discussed in a subsequent paper.

* *The reader should be careful not to confuse the British Sovereign with the Royal Sovereign to which it bears no relation.*

The *Fragaria vesca* plants were from a clone obtained from East Malling, England, by Dr. G. H. Berkeley, of the Plant Pathology Laboratory, St. Catharines, Ont.

The method of inoculation was by stolon grafts, according to the technique first described by Harris (2).

Graft Transmission of Yellows from Marshall to British Sovereign

Late in July, 1948, two British Sovereign plants were grafted to a Marshall plant severely affected with yellows. These British Sovereign plants had been growing undisturbed for several months and a number of runner plants from each were already established. The grafts were made a few inches behind the actively growing stolon tip, which continued to grow and form runner plants. In some cases the grafted stolon arose directly from the parent plant; in others a rooted runner plant intervened. In all, five such grafts were made. One graft on each plant made a definite union; three were uncertain. The grafts were left intact for two months before the connections with the diseased Marshall plant were severed, and the stolons connecting the British Sovereign runner plants were left intact for another three months, so that there was ample opportunity for migration of the virus through all parts of both clones, not only to the extremities of the grafted stolons, but also through the parent plants, into the supplementary stolons (those which had not actually been grafted).

Toward the end of September, runner plants on the successfully grafted stolons began to show symptoms (Pl. I, Figs. 1 and 2). In these plants there was a tendency for the petioles of the younger leaves to reflex toward the ground and for autumnal coloration to be premature. There was no visible effect on the parent plants, the plants on grafted stolons where union had been uncertain, or the plants on the supplementary stolons during the fall of 1948.

Early in January, 1949, the runner plants, both those on the grafted stolons and on the supplementary stolons, were brought into the greenhouse. From the time they began to put forth new growth, abnormalities were observed.

The earliest effect, noticeable after one month in the greenhouse, was a slight marginal chlorosis of the leaves, narrowness at the base of the leaflets, reflexing of the petioles toward the ground, and a dwarfing of the plant. As a rule, this effect was most pronounced on the distal plant of each stolon; less noticeable on the older runner plants. The terminal plants and most of the plants produced on the two successfully grafted stolons put out only three or four small leaves, then seemed to remain in a state of suspended development for several months (Pl. I, Fig. 4). Most of these extremely dwarfed individuals were dead by the end of May. Many of the plants, however, were less severely affected (Pl. I, Fig. 5), although they all showed a tendency to a flattened rather than an upright habit of growth, and, in these, degeneration was more gradual.

After five months in the greenhouse, the check plants were still green and flourishing (Pl. I, Fig. 6), but most of the surviving inoculated plants were much reduced in vigor (Pl. I, Fig. 7). The leaves were smaller than normal, the leaflets tended to be narrow at the base, the petioles were flattened toward the ground, and there was some premature reddening of the older leaves. In some cases this premature coloring consisted of a gradual reddening of the leaves, with the veins remaining green, but in others the veins colored first, creating a vivid pattern against the green leaf.

Yellowing of the leaf margins, characteristic of the disease on the Marshall variety, was either very slight or entirely absent.

Some of these plants died during the summer of 1949. A few more died during the following winter. Others survived till 1950, and even produced a small crop before they succumbed. Still others, though exceedingly small and debilitated, were still alive at the end of 1950.

Graft Transmission of Yellows from Marshall to *Fragaria vesca*

A large *Fragaria vesca* plant, with two stolons having several plants on each, was grafted to a yellows-infected Marshall plant. The graft was made between the first and second plant on one of these stolons.

The effect was rapid and severe. The first symptom was a flattening toward the ground of all leaves that were already formed when the graft was made. This effect was evident within a month on the first runner plant beyond the point of grafting; soon afterwards on the plant between the graft and the parent; and a little later on the parent plant itself. At the same time the color of these leaves changed from the normal bright green to a duller more yellow-green. These early symptoms were followed by a premature reddening and the eventual death of these older leaves.

The leaves that developed shortly after the first symptoms appeared were smaller than normal and displayed some crinkling of the surface and yellowing of the margins (Pl. II, Fig. 2). All of the later leaves were extremely small – less than an inch across – and markedly chlorotic at the margin. However, the veins remained green and there was little or no mottle, crinkle, or leaf distortion (Pl. II, Figs. 3, 4, and 5).

The effect of yellows on British Sovereign inoculated by grafting to a diseased Marshall plant.

Fig. 1. Control plant.
Fig. 2. Runner plant from grafted stolon two months after inoculation showing reflexing of younger petioles.
Fig. 3. Control plant on resumption of growth the following spring.
Fig. 4. Runner plant from grafted stolon on resumption of growth in the spring, showing extreme dwarfing, narrowness at base of leaflets, and reflexing of petioles.
Fig. 5. Runner plant from supplementary stolon on resumption of growth in the spring, showing narrowness at base of leaflets and reflexing of petioles.
Fig. 6. Control plant four months later. Note the upright type of growth.
Fig. 7. Inoculated plant four months later, showing reduction in leaf size and flattened type of growth.

PLATE I

The effect of yellows on *Fragaria vesca* inoculated by grafting to a diseased Marshall plant.

FIG. 1. Control plant.

FIG. 2. Parent plant of grafted clone two months after grafting, showing flattening of the older leaves, crinkling and twisting of younger leaves, and hooking back of runner tips.

FIG. 3. Parent plant three months after grafting, showing extreme dwarfing of the younger leaves.

FIG. 4. Crown detail of parent plant three months after grafting, showing very small younger leaves, yellow at their margins.

FIG. 5. Crown detail of runner plant from grafted clone three months after grafting. Two older leaves, formed before the graft was made, are flattened toward the ground; all leaves subsequently produced are minute, though not distorted.

FIG. 6. Crown detail of runner plant from grafted clone six months after grafting. Older leaves are dead; younger leaves are dying.

The leaf symptoms were accompanied by a hooking back of the stolon tips (Pl. II, Fig. 2), the death of some of the younger stolons, and finally complete cessation of stolon production.

The development of the extremely dwarfed leaves in the center of the crown and the gradual dying of older leaves eventually reduced the plants to mere tussocks (Pl. II, Fig. 6). After the plants reached this stage, there was no appreciable change in the symptom picture, but the plants became more and more debilitated; several died during the summer, and none survived the following winter.

Discussion

The results of the grafting experiments with British Sovereign leave little doubt that this variety is susceptible to the yellows disease, and can be expected to degenerate if the disease becomes established in the stock.

The results of the experiment with *Fragaria vesca* show that this species is equally as susceptible to the yellows of the Pacific Coast as Harris and King (5) have shown it to be to the yellow-edge disease of Great Britain. The question of whether the two diseases, yellows and yellow-edge, are identical will be discussed in a subsequent paper of this series.

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THE SOURCE OF RESISTANCE AND THE INHERITANCE OF REACTION TO 12 PHYSIOLOGIC RACES OF STEM RUST, *PUCCINIA GRAMINIS AVENAE* (ERIKSS. AND HENN.)[1]

By J. N. Welsh[2] and T. Johnson[3]

Abstract

Resistance to 12 races of oat stem rust was unexpectedly obtained from three crosses, Hajira–Jostrain, Hajira–Richland, and Hajira–Banner, between the years 1932 and 1940. Recent investigations have shown that the Hajira parent was the source of this resistance, as 10% of plants selected from this variety were found to be highly resistant. Inheritance studies have shown that, in some crosses involving this source of resistance, two main genes are involved and in others only a single main gene. A probable explanation is that the highly resistant plants in Hajira may have differed genetically. The studies also reveal that the Hajira type of resistance to race 8, either in the seedling or adult stage, is indicative of resistance to the 12 races that occur in Canada and the United States.

Introduction

Prior to the distribution of resistant varieties in 1937 stem rust caused heavy losses to the oat crop in Canada. At that time 10 races of this rust (races 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10) were known to occur in Canada. Since then three more races, 11, 12, and 13, have been collected, making a total of 13 races that are known at the present time to occur in both Canada and the United States. Race 9 was identified by Gordon (3) but since that time cultures of this race have not been collected in Canada. The rust resistant varieties grown commercially in Canada are resistant to races 1, 2, 3, 5, 7, and 12 only. Prior to 1943 rust infections were caused mainly by races 1, 2, and 5 as annual rust surveys demonstrated that these three races comprised 95% of the rust specimens collected. The commercially grown varieties were resistant under field conditions until 1943, when races 8, 10, and 11 appeared in epidemic proportions. These races have appeared in each rust year since then and their presence has reduced yields considerably, especially in late sown crops. In view of this fact the production of varieties resistant to these three races and to other less prevalent ones that may appear in the future in epidemic proportions, was essential. Varieties having resistance to all known races of stem rust have been produced at the Dominion Laboratory of Cereal Breeding, Winnipeg, Canada. The purpose of this paper is to report on the source of this resistance and on the inheritance of reaction to the different races.

Materials and Methods

Materials

The varieties used as parents in these studies, together with their seedling reaction to 12 races of stem rust are given in Table I. The origin of certain

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of these varieties is as follows. Hajira, C.I. 1001, was obtained from El-Hajira, Egypt, in 1904 as an unnamed variety, by the Department of Agriculture, Pretoria, Union of South Africa and named after the place from which it was obtained. It was introduced into the United States from South Africa

TABLE I

SEEDLING REACTION TO 12 RACES OF STEM RUST OF THE VARIETIES USED AS PARENTS

Varieties	R.L. No.	Physiologic races											
		1	2	5	3	7	12	8	10	11	4	6	13
Hajira	559	R	R	R	R	R	R	S	S	S	S	S	S
Monarch strain	560	R	R	R	R	R	R	S	S	S	S	S	S
Richland	172	R	R	R	R	R	R	S	S	S	S	S	S
Ajax	1114	R	R	R	R	R	R	S	S	S	S	S	S
Beacon	58	R	R	R	R	R	R	S	S	S	S	S	S
Victory × Hajira	1114.1	R	R	R	R	R	R	S	S	S	S	S	S
White Russian	177	MR	MR	MR	S	S	S	MR	MR	MR	S	S	S
Jostrain	561	R	S	MR	R	S	MR	S	MS	R	R	S	MR
Hajira × Banner	524	R	R	R	R	R	R	R	R	R	R	R	R
Canuck	811	R	R	R	R	R	R	R	R	R	R	R	R
Victoria × R.L. 524	1272	R	R	R	R	R	R	R	R	R	R	R	R
Victory × R.L. 1272	1681	R	R	R	R	R	R	R	R	R	R	R	R
Garry	1692	R	R	R	R	R	R	R	R	R	R	R	R
Victory	159	S	S	S	S	S	S	S	S	S	S	S	S

in 1919 and for a time was wrongly designated as Heigira Rustproof, Heijira, Heijara, and Hajera (5). Heigira Strain was the spelling used by Bailey (1) and Welsh (8). According to Bailey (1), Monarch Strain is a selection from Monarch selection C.I. 1879 and Jostrain (formerly designated as Joanette Strain) is a selection from Joanette C. I. 1880; Richland is a selection from Kherson, Stanton *et al.* (7); White Russian is thought to have come from

TABLE II

CROSSES, YEAR MADE, GENERATIONS, AND STEM RUST RACES STUDIED

Crosses	Year crosses made	Generations studied	Stem rust races used
Hajira × Jostrain	1926	F^3 F_6	All races
Victoria × R.L. 524	1930	F_9	All races
Hajira × Richland	1935	F_3	All races
Jostrain × Hajira	1937	F_3	Race 6
Monarch Strain × Hajira	1937	F_3	Race 6
Jostrain × Richland	1937	F_3	Race 6
Jostrain × White Russian	1937	F_3	Races 5, 6, 7, 8
Beacon × R.L. 1681	1945	F_2 F_3	All races
R.L. 1114.1 × R.L. 1681	1945	F_2 F_3	All races
Ajax × Garry	1945	F_1 F_2 F_3	All races
Canuck × Victory	1946	F_1 F_2 F_3	All races

Russia; Victoria is of South American origin, while Victory is a selection from the German variety Milton, Derick *et al.* (2). The parentage of the other varieties is as follows: Ajax (Victory × Hajira); Beacon (Gold Rain – Alaska) × (Legacy – Victoria) × (R.L. 453 – Vanguard); Canuck (Hajira × Jostrain); and Garry (Victory × R.L. 1272). The crosses studied are given in Table II.

Methods

Experiments were conducted both in the greenhouse and in the field. In the greenhouse approximately 30 seeds per pot were sown when carrying on genetic studies and 15 seeds when testing the reaction of lines to a number of races. Inoculations were made by hand and two types of incubation chambers were used. A small, round chamber made of galvanized iron and provided with a glass top was used when testing a relatively few lines to the different races. In the inheritance studies, in which large populations were inoculated with a single race, a cotton covering supported on a wooden frame 6 ft. long, 32 in. wide, and 12 in. high was used. On completion of the inoculations the seedlings and the cotton covering were sprayed with water, the latter being kept moist during the day by frequent sprayings. The temperature in the greenhouse was lowered immediately after inoculation, usually dropping as low as 50° F. during the night. The incubation period lasted from 16 to 20 hr. depending on the time the inoculations were completed, as the cotton covering was always removed the following morning. Excellent infections were obtained by this method.

In recording the rust reactions, the seedlings in each pot were read individually. The symbols used were R, for the resistant 1 and 2 pustule types, R- for the resistant 2 + type, and S for the susceptible 4 type of pustule.

In the field, seed of each F_1 plant was sown separately in F_2 for all crosses, with the exception of Beacon × R.L. 1681 and Ajax × Garry. The F_1 plants of these two crosses were unintentionally bulked when harvested. Each F_2 line was sown in a plot consisting of two 10-ft. rows, with the seeds spaced approximately 2½ in. apart in the row. A single row of a susceptible variety was sown along the ends of the plots to serve as an infection center. An artificial epidemic of stem rust was created by dusting spores on the infection rows in the evenings when a dew was expected. Prior to 1944 the artificial rust epidemics were confined to the common races 1, 2, and 5, and races 3, 7, and 12 to all of which the variety Richland is resistant. This was done because much of the plant breeding material under study had the Richland type of resistance, and the presence of other races would have militated against its selection. Since 1944 all available races have been included in these epidemics.

Resistance to 12 Races of Stem Rust

Resistance to 12 races of stem rust was obtained from three crosses, Hajira – Jostrain, Hajira – Richland, and Hajira – Banner between the years 1932-1940. Welsh (9) obtained resistance to races 1, 2, 3, 4, 5, 6, 7, 8, and 10, the

only ones identified at that time, from a cross between Hajira and Joanette Strain. The latter variety is referred to as Jostrain in the present paper. Later, Newton *et al.* (6) inoculated lines of this cross with the same nine races, in addition to three more recently identified ones, 11, 12, and 13, and found them to be resistant to all 12 races.

In view of the above results, other crosses were made between resistant varieties with the object of determining from what other varietal combinations similar resistance could be obtained. During the three years 1938-1940, F_3 random lines of the following five crosses, Hajira × Richland, Jostrain × Hajira, Jostrain × Richland, Monarch Strain × Hajira, and White Russian × Jostrain, were inoculated in the seedling stage with race 6. Resistant lines were observed in the Hajira × Richland cross only. In this cross the lines of two families out of seven tested produced resistant and susceptible seedlings, while those in the remaining five families were susceptible. Under field conditions, in which the artificial rust epidemic included all 12 races, a number of resistant, segregating, and susceptible lines of this cross produced reactions similar to those obtained in the greenhouse with race 6. In a later test, a number of the resistant lines were inoculated with all 12 races and were found to be resistant to all of them.

The source of the resistance obtained in the crosses Hajira × Jostrain and Hajira × Richland was not determined until 1939. In that year resistance to the 12 races was unexpectedly obtained from a cross between Victoria and R.L. 524. The latter parent is from the Hajira × Banner cross and was presumed to be resistant to races 1, 2, 3, 5, 7, and 12, only. Until 1939, all lines of the cross Victoria × R.L. 524 were resistant in the field, where only the above races had been present. In that year certain lines were observed to be susceptible and rust samples taken from them were identified as race 8, indicating that all lines that were free from rust were resistant to this race. Tests later conducted showed that R.L. 524 and the lines that were resistant in the field were resistant to all 12 races, while the lines that were susceptible in the field were resistant to races 1, 2, 3, 5, 7, and 12, but susceptible to the other six races.

The fact that R.L. 524 and all lines previously found to be resistant to all races had Hajira in their parentage, suggested the possibility that this variety contained a few plants that were resistant to all of the races and that when crosses were made, some of these plants were accidently pollinated.

Reaction of Hajira Selections to 12 Races of Stem Rust

To explore the possibility that Hajira contained plants resistant to race 8, 400 Hajira plants selected at random were inoculated in the seedling stage with this race. Of this number 42 were resistant, 26 segregated, and 332 were susceptible. All seedlings tested displayed either the resistant type 1 or the susceptible type 4 pustule. The pustule types are shown in Fig. 1.

PLATE I

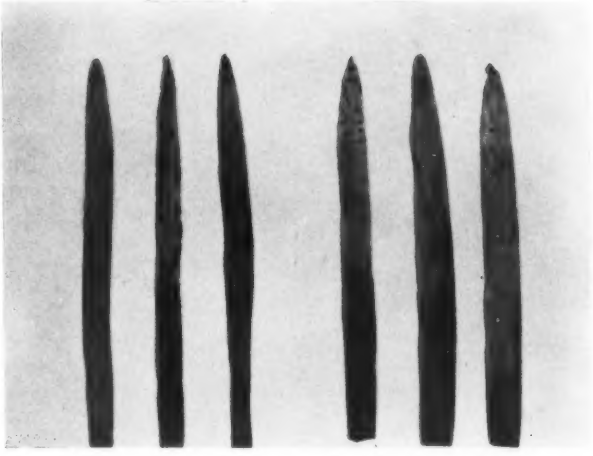

FIG. 1. Seedling reaction of susceptible (left) and resistant (right) selections of Hajira to race 8 of oat stem rust.

In 17 of the segregating selections, the resistant seedlings predominated and in nine of them the susceptible ones were more numerous. A number of the resistant, susceptible, and segregating selections were later inoculated with all 12 races in the seedling stage. These were not all tested at the same time, as two lots were inoculated in 1945 and one in 1946. The reactions of a certain number of them as well as the number of resistant and susceptible seedlings observed in the original test with race 8, are given in Table III.

TABLE III

RELATIONSHIP BETWEEN THE INITIAL SEEDLING REACTION OF HAJIRA SELECTIONS TO RACE 8 AND THE SEEDLING REACTION TO ALL 12 RACES

Sel. No.	Initial reaction to race 8		Physiologic races											
	Res. seedlings	Susc. seedlings	1	2	3	5	7	12	4	6	8	10	11	13
16	15	0	R	R	R	R	R	R	R	MR	R	R	R	R
18	20	0	R	R	R	R	R	R	R	MR	R	R	R	R
20	18	0	R	R	R	R	R	R	R	MR	R	R	R	R
29	16	0	R	R	R	R	R	R	R	MR	R	R	R	R
34	17	0	R	R	R	R	R	R	R	MR	R	R	R	R
44	18	0	R	R	R	R	R	R	R	MR	R	R	R	R
49	16	0	R	R	R	R	R	R	R	MR	R	R	R	R
26	15	3	R	R	R	R	R	R	R;S	S;X*;R	R;S	R;S	R;S	R;S
51	18	2	R	R	R	R	R	R	R	R	R	R	R	R
68	15	2	R	R	R	R	R	R	R;S	S;X;R	R;S	R;S	R;S	R;S
243	10	3	R	R	R	R	R	R	R;S	S;X;R	R;S	R;S	R;S	R;S
353	17	3	R	R	R	R	R	R	R;S	S;X	R	R;S	R;S	R;S
198	1	16	R	R	R	R	R	R	S	S	S;R	S;R	S;R	S
220	2	16	R	R	R	R	R	R	S;R	S;R	S	R;S	S;R	S;R
273	2	16	R	R	R	R	R	R	S	S	S	S;R	S;R	S
321	1	14	R	R	R	R	R	R	S;R	X	S	S	S;R	S;R
130	0	21	R	R	R	R	R	R	S	S	S	S	S	S
143	0	20	R	R	R	R	R	R	S	S	S	S	S	S
144	0	17	R	R	R	R	R	R	S	S	S	S	S	S
150	0	19	R	R	R	R	R	R	S	S	S	S	S	S
156	0	19	R	R	R	R	R	R	S	S	S	S	S	S

*X = *Indeterminate reaction.*

The data show that all selections were resistant to races 1, 2, 3, 5, 7, and 12, but that they varied in their reaction to the other six. For purposes of discussion, therefore, the races may be classified into two groups, Group I representing races 1, 2, 3, 5, 7, and 12, and Group II races 4, 6, 8, 10, 11, and 13. The selections that were resistant to race 8 were resistant to those in Group II, with the exception of race 6, to which they were classified as moderately resistant, as the pustule types produced by this race varied in the different tests. This race produced a type 1 pustule in the 1945 tests and a type 4- in 1946. Experiments demonstrated that at 61° F. this race produced a resistant type of pustule, and at 75° F. a susceptible one. This effect of temperature

on reaction to race 6 was originally recorded by Welsh (9) and more recently by Kehr and Hayes (4). In the selections segregating to race 8 the ratio of resistant to susceptible seedlings for the races in Group II was the same as in the original test with race 8, with a few exceptions. The exceptions are perhaps due to the use of too few seedlings in the tests concerned. The selections that were susceptible to race 8, on the other hand, were susceptible to the races in Group II.

There are, therefore, three types of resistant plants in Hajira. Approximately 10% are resistant to all 12 races, 7% are resistant to Group I races and segregate to the races in Group II, and 83% are resistant to Group I and susceptible to Group II. Apparently it was chance pollination with the more highly resistant ones that was responsible for the production of varieties resistant to all 12 races in crosses of Hajira with Jostrain, Banner, and Richland. In an attempt to explain the presence of the highly resistant plants in Hajira it is necessary to refer to an earlier work by Bailey (1) who made the original selections in his search for differential hosts. He found in Hajira, referred to by Bailey as Heigira Rustproof (C.I. 1001), that approximately five per cent of the plants were susceptible to the culture of stem rust used. The resistant plants were bulked and used as differential host material. This bulked material, designated by Bailey as Heigira Strain, was resistant to races 1, 2, 3, and 5, but susceptible to race 4. At that time only races 1, 2, and 5 had been identified in Canada and the United States; races 3 and 4 were from cultures of Swedish origin.

The seed of the Hajira used in the present experiments was from the bulked plant selections made by Bailey (1). It would appear that that variety contained not only plants resistant to a few races, but also some that were resistant to 12 races. The presence of plants resistant to the races in Group I and segregating to those in Group II could be due to natural crossing between the types of resistant plants.

Inheritance of Stem Rust Reaction

As the previous results demonstrated that Hajira was the source from which resistance to the 12 races of stem rust was derived, a study was conducted to determine the inheritance of this type of resistance. The crosses used were Beacon × R.L. 1681, R.L. 1114.1 × R.L. 1681, Ajax × Garry, Canuck × Victory, and Jostrain × White Russian. The genetic data for the Beacon × R.L. 1681 cross is not recorded as an unusual ratio was obtained in F_3, which could be attributed to an improper random selection in F_2. The F_2 of this cross was harvested in 1946, a year in which germination was very poor, with the result that many plants failed to produce viable seeds. All of the resistant parents, with the exception of Jostrain and White Russian, have Hajira in their parentage. The cross Jostrain × White Russian was included in these studies to compare the inheritance of the White Russian resistance to race 8 with the Hajira type of resistance to this race.

Experiments were conducted in the field and in the seedling stage in the greenhouse. In the field, the F_2 plants were separated into two classes, resistant and susceptible. In the greenhouse, random F_3 lines of all five crosses were inoculated with race 8 and, in addition, random F_3 lines of the Canuck × Victory cross were inoculated with race 11. The following rust classes were recorded: homozygous resistant (R) or (R-); heterozygous resistant (R,R-); segregating (R,R-,S) and (R-,S); and susceptible (S). Later, a certain number of lines that were classed as resistant, segregating, and susceptible to race 8 were inoculated with all 12 races in the seedling stage. To determine the relation between seedling reaction to race 8 and field reaction to all races, F_3 populations of three crosses were grown in the field. Eighty F_3 lines of Beacon × R.L. 1681 were grown in 1947, and 153 lines of the R.L. 1114.1 × R.L. 1681 cross and 173 of the Ajax × Garry cross in 1948. F_1 reactions were taken on the Ajax × Garry and Canuck × Victory crosses only. The F_1 plants of the former cross were resistant to race 8 in the seedling stage, while those of the latter cross were resistant in the field and also to races 4, 6, 8, and 13 to which they were inoculated in the seedling stage in the greenhouse. Moreover, in a number of more recent crosses, involving the Hajira source of resistance, all F_1 plants were resistant under field conditions. From these observations and from the data to be discussed it is apparent that resistance is dominant in all crosses.

F_2 Field Studies

The mode of inheritance in F_2 for three crosses, R.L. 1114.1 × R.L. 1681, Ajax × Garry, and Canuck × Victory, is given in Table IV. The results

TABLE IV

SEGREGATION IN F_2 FOR RESISTANCE AND SUSCEPTIBILITY TO ALL RACES OF STEM RUST IN THE FIELD FOR THREE CROSSES AND THE CALCULATION OF GOODNESS OF FIT TO A 3 : 1 RATIO

Ratio	Rust class	Observed	Calculated	χ^2	P values
R.L. 1114.1 × R.L. 1681					
3	R	256	250.50	—	—
1	S	78	83.50	0.48	0.50
Ajax × Garry					
3	R	137	147.75	3.13	0.05
1	S	60	49.25	—	—
Canuck × Victory					
3	R	665	672.00	0.29	0.70–0.50
1	S	231	224.00	—	—

indicate that in all three crosses the inheritance is governed by a single main gene. Two families of the R.L. 1114.1 × R.L. 1681 cross, totalling 334 plants, were classified in F_2. In one family 133 plants were classified as resistant and 36 as susceptible, while in the second family 123 were resistant and 42 susceptible. Good agreements were obtained to a 3 : 1 ratio for each family, as evidenced by a P value of approximately 0.25 for one of them and 0.90 for the other. The data for both families combined are given in Table IV in which a P value of 0.50 indicates a good agreement to a single-factor hypothesis.

The F_2 population of the Ajax × Garry cross, consisting of 197 plants, was the progeny of F_1 plants that were bulked at harvest time owing to an oversight. Of the number classified in F_2, 137 were resistant and 60 susceptible. The P value of 0.05 indicates a fit that approaches a 3 : 1 ratio.

Twenty-seven F_2 families of the cross Canuck × Victory, consisting of 896 plants, were classified in F_2 and of this number 665 were resistant and 231 susceptible. A P value of approximately 0.60 suggests a satisfactory fit to a 3 : 1 ratio.

The F_2 of the cross Jostrain × White Russian was grown in 1939 at which time the artificial rust epidemic did not include all races; consequently, data for this cross were not available under comparable conditions.

F_3 Greenhouse Studies with Race 8

The F_2 field rust classes and the corresponding F_3 seedling classification to race 8 are given in Table V for the four crosses mentioned above. The data show that the proportion of resistant lines is greater in the cross R.L. 1114.1 × R.L. 1681 than in the other three crosses. Furthermore, the (R-) (R,R-), and (R-,S) classes are more numerous in this cross than in the Ajax × Garry or Canuck × Victory crosses. The inheritance in this cross appears to be more complicated than that suggested by the F_2 field studies in which a ratio of three resistant to one susceptible plant was observed. In the other crosses the resistant and susceptible classes are approximately equal indicating a more simple type of inheritance. In the Jostrain × White Russian cross it is to be noted that only three rust classes were observed: resistant (R-), segregating (R-,S), and susceptible (S). The genetic analyses for the four crosses are given in Table VI.

Two F_3 families of the R.L. 1114.1 × R.L. 1681 cross were inoculated with race 8. In one family consisting of 165 lines out of 169 plants classified in F_2, 74 were resistant, 84 segregated, and 7 were susceptible. In the second family, which consisted of 164 lines out of 165 plants classified in F_2, 71 were resistant, 82 segregated, and 11 were susceptible. Good fits to a 7 : 8 : 1 ratio were obtained for each family as evidenced by P values of 0.50 and 0.80, respectively. The data for both families combined are given in Table VI. A good agreement was obtained to a 7 : 8 : 1 ratio as shown by a χ^2 value of 0.34 and a P value of 0.80. A good fit to 15 : 1 ratio is obtained when the resistant

TABLE V

F_2 FIELD CLASSIFICATION TO ALL RACES OF STEM RUST AND F_3 SEEDLING CLASSIFICATION TO RACE 8 IN THE GREENHOUSE, FOR FOUR CROSSES

F_2 field classification	F_3 seedling classification to race 8							Total F_2 plants
	Resistant			Segregating			Susceptible	
	R	R-	R,R-	R,R-,S	R,S	R-,S	S	
R.L. 1114.1 × R.L. 1681								
R	102	3	22	108	12	6	—	253
S	—	18	—	5	—	35	18	76
Total F_3 lines	102	21	22	113	12	41	18	329
Ajax × Garry								
R	38	—	2	69	23	—	—	132
S	—	—	—	3	3	—	51	57
Total F_3 lines	38	—	2	72	26	—	51	189
Canuck × Victory								
R	111	—	3	57	139	1	—	311
S	—	—	—	2	10	4	107	123
Total F_3 lines	111	—	3	59	149	5	107	434
Jostrain × White Russian								
Total F_3 lines	—	84	—	—	—	167	76	327

and segregating classes are combined, as indicated by a P value of approximately 0.60. The F_3 results with this cross, therefore, are not in agreement with the single-factor hypothesis suggested by the F_2 field data.

In the three other crosses, on the other hand, the F_3 data are in agreement with the single-factor hypothesis established in F_2. In the Ajax × Garry cross, 189 lines from the 197 plants classified in F_2 were inoculated in F_3, and of this number 40 were resistant, 98 segregated, and 51 were susceptible. Twelve families of the 27 classified in F_2 for the Canuck × Victory cross were inoculated in F_3. Four hundred and thirty-four lines were tested, of which 114 were resistant, 213 segregated, and 107 were susceptible. In the cross Jostrain × White Russian, 327 random lines were inoculated, and of this number, 84 were resistant, 167 segregated, and 76 were susceptible. For the three crosses, P values of approximately 0.40, 0.80, and 0.70, respectively, for two degrees of freedom, indicate good fits to a 1 : 2 : 1 ratio.

TABLE VI

INHERITANCE OF REACTION TO RACE 8 IN THE SEEDLING STAGE OF F_3 LINES OF FOUR CROSSES AND THE CALCULATION OF THE GOODNESS OF FIT TO A 7 : 8 : 1 RATIO IN ONE CROSS AND A 1 : 2 : 1 RATIO IN THREE CROSSES

Ratio	Rust class	Observed	Calculated	χ^2	P values
R.L. 1114.1 × R.L. 1681					
7	R	145	143.94	—	—
8	Seg	166	164.50	—	—
1	S	18	20.56	0.34	0.80
Ajax × Garry					
1	R	40	47.25	—	—
2	Seg	98	94.50	—	—
1	S	51	47.25	1.54	0.50–0.30
Canuck × Victory					
1	R	114	108.50	—	—
2	Seg	213	217.00	—	—
1	S	107	108.50	0.37	0.90–0.80
Jostrain × White Russian					
1	R	84	81.75	—	—
2	Seg	167	163.50	—	—
1	S	76	81.75	0.54	0.80–0.70

F_3 Greenhouse Studies with Race 11

In the Canuck × Victory cross, as shown in Table X, F_3 lines, previously classed as resistant and susceptible, respectively, in F_2, were inoculated with all 12 races. For the lines classed as susceptible in F_2 the reactions to races 1, 4, and 11 differed from those for the other races. As Jostrain, one of the parents of Canuck, is resistant to these three races (Table I), the reactions suggest that the Jostrain parent contributed a factor for resistance to them, in addition to the resistance of the Hajira parent.

To test this hypothesis six F_3 families of the Canuck × Victory cross, totalling 224 plants, were inoculated with race 11. Of this number 103 were resistant, 108 segregated, and 13 were susceptible. On the basis of a 7 : 8 : 1 ratio, a P value of 0.80 for two degrees of freedom suggests a good agreement with this two factor hypothesis. It is apparent, therefore, that Canuck derived an additional factor for resistance to race 11 from the Jostrain parent. Although races 1 and 4 were not studied it is assumed, on the basis of the similarity of their behavior to that of race 11 in Table X, that Jostrain has

also contributed a factor for resistance to these two races. Furthermore, observations over a period of years indicate that Canuck was, in general, more resistant than varieties with only the Hajira type of resistance to all races.

Seedling Reaction to 12 Races of Stem Rust

The seedling reactions of F_3 lines, previously classified as resistant and susceptible respectively in F_2 to 12 races of stem rust, are given for four crosses in Tables VII, VIII, IX, and X. The data in Table VII, for the Beacon × R.L. 1681 cross, show that lines which were resistant in the field were, for the most part, resistant to the races in Group I, namely 1, 2, 3, 5, 7, and 12. A number were resistant to all of the races in the second Group, 4, 6, 8, 10, 11, and 13, while others gave a segregating reaction to the races in this group. Three lines gave reactions contrary to the above results. Line 113 gave an (R,R-) reaction to eight of the races and lines 34 and 40 gave (R,R-) and segregating reactions to certain races in Group I.

TABLE VII

SEEDLING REACTION TO 12 RACES OF STEM RUST OF F_3 LINES OF THE CROSS BEACON × R.L. 1681, PREVIOUSLY CLASSIFIED IN F_2 AS RESISTANT AND SUSCEPTIBLE UNDER FIELD CONDITIONS

F_3 line No.	F_2 field reaction	Physiologic races of stem rust											
		1	2	3	5	7	12	4	6	8	10	11	13
1	R	R	R	R	R	R	R	R	R	R	R	R	R
7	R	R	R	R	R	R	R	R	R	R	R	R	R
10	R	R	R	R	R	R	R	R	R	R	R	R	R
27	R	R	R	R	R	R	R	R	R	R	R	R	R
113	R	R,R-	R,R-	R,R-	R,R-	R,R-	R,R-	R,R-	R,R-	R	R	R	R
123	R	R	R	R	R	R	R	R	R	R	R	R	R
125	R	R	R	R	R	R	R	R	R	R	R	R	R
130	R	R	R	R	R	R	R	R	R	R	R	R	R
4	R	R	R	R	R	R	R	Seg*	Seg	Seg	Seg	Seg	Seg
20	R	R	R	R	R	R	R	Seg	Seg	Seg	Seg	Seg	Seg
25	R	R	R	R	R	R	R	Seg	Seg	Seg	Seg	Seg	Seg
42	R	R	R	R	R	R	R	Seg	Seg	Seg	Seg	Seg	Seg
34	R	R,R-	R,R-	Seg	R,R-	R	Seg	Seg	Seg	Seg	Seg	Seg	Seg
40	R	R,R-	R	Seg	R	R	R	Seg	Seg	Seg	Seg	Seg	Seg
164	S	R	R	R-	R-	R-	R	S	S	S	S	S	S
166	S	R	R	R-	R	R-	R	S	S	S	S	S	S
175	S	R	R	R-	R	R-	R	S	S	S	S	S	S
193	S	R	R	R-	R-	R-	R	S	S	S	S	S	S
171	S	R	R	S	R-	R-	R-	R-	R-,S	R-	R-	R	R-
186	S	R	R	S	S	S	S	R-	R-,S	R-	R-	R-	R-
187	S	R	R	S	R-	S	S	R-	R-,S	R-	R-	R-	R-
169	S	R	R	R-	Seg	R-	R-	R-,S	S	Seg	Seg	Seg	R-,S
170	S	R	R	R-,S	Seg	R-	R-,S	R-,S	S	R-,S	S	R-,S	R-,S
178	S	R	R	R-,S	R-,S	R-	Seg	R-,S	R-,S	R-,S	R-,S	R-,S	R-,S

*-Seg = *highly resistant seedlings present.*

The lines that were susceptible in the field varied in their reaction to the races in both groups. A few were resistant to races 1, 2, 3, 5, 7, and 12, and susceptible to the other six races; others gave resistant, segregating, and susceptible reactions to certain races in both groups, with the resistant types being mostly of the R- class. All of these lines, however, were resistant to races 1 and 2.

The data in Table VIII, for the cross R.L. 1114.1 × R.L. 1681, are similar to those in Table VII with the exception that all of the lines classed as susceptible in F_2 were not resistant to races 1 and 2.

TABLE VIII

SEEDLING REACTION TO 12 RACES OF STEM RUST OF F_3 LINES OF THE CROSS R.L. 1114.1 × R.L. 1681, PREVIOUSLY CLASSIFIED IN F_2 AS RESISTANT AND SUSCEPTIBLE UNDER FIELD CONDITIONS

F_3 line No.	F_3 field reaction	F_3 seedling reaction to 12 races											
		1	2	3	5	7	12	4	6	8	10	11	13
167	R	R	R	R	R	R	R	R	R	R	R	R	R
175	R	R	R	R	R	R	R	R	R	R	R	R	R
196	R	R	R	R	R	R	R	R	R	R	R	R	R
199	R	R	R	R	R	R	R	R	R	R	R	R	R
216	R	R	R	R	R	R	R	R	R	R	R	R	R
217	R	R	R	R	R	R	R	R	R	R	R	R	R
277	R	R	R	R	R	R	R	R	R	R	R	R	R
189	R	R	R	R	R	R	R	Seg	Seg	Seg	Seg	Seg	Seg
195	R	R	R	R	R	R	R	Seg	Seg	Seg	Seg	Seg	Seg
198	R	R	R	R	R	R	R	Seg	Seg	Seg	Seg	Seg	Seg
274	R	R	R	R	R	R	R	Seg	Seg	Seg	Seg	Seg	Seg
174	R	R	R	Seg	R	R	Seg	Seg	Seg	Seg	Seg	Seg	Seg
176	R	R	R	Seg	Seg	R	R	Seg	Seg	Seg	Seg	Seg	Seg
177	R	R	Seg	Seg	Seg	R	Seg	R	Seg	R	R	R	R
194	R	Seg	R	Seg	Seg	R	Seg	Seg	Seg	Seg	Seg	Seg	Seg
310	S	R	R	R	R	R	R	S	S	S	S	S	S
315	S	R	R	R	R	R	R	S	S	S	S	S	S
328	S	R	R	R	R	R	R	S	S	S	S	S	S
161	S	R	R	R	R	R	R	S	S	S	S	S	S
213	S	R	R	R	R	R	R	S	S	S	S	S	S
313	S	R	R	R	R	R	R	S	S	S	S	S	S
207	S	R,R-	Seg	Seg	Seg	R,R-	Seg	Seg	S	Seg	R-,S	Seg	Seg
309	S	R,R-	Seg	Seg	Seg	R,R-	Seg	Seg	S	Seg	Seg	Seg	S
208	S	R,R-	R-	Seg	R	R,R-	Seg	S	S	Seg	Seg	Seg	R-,S
211	S	R,R-	R-,S	S	R-,S	S	S	R	S	R-	R-	R-	R-
320	S	Seg	Seg	Seg	Seg	Seg	R	Seg	S	Seg	Seg	Seg	Seg
326	S	R	Seg	Seg	Seg	R,R-	Seg	R-,S	S	Seg	Seg	Seg	S
327	S	R	Seg	Seg	Seg	Seg	Seg	R-,S	S	Seg	Seg	Seg	R-,S
209	S	R-	S	S	S	R-	S	R-	S	R-	R	R-	R-

In the cross Ajax × Garry, as indicated in Table IX, all lines whether classed as resistant or susceptible in F_2, were resistant to the races in Group I. Of those that were classed as resistant in F_2, some were resistant to all races,

while others segregated in their reaction to the races in Group II. The lines that were classed as susceptible in F_2 were either susceptible to all of the races in Group II or segregated in their reaction to this group of races.

The F_3 lines of the Ajax × Garry cross that were inoculated in the seedling stage with race 8, were also inoculated with races 2 and 7, representatives of Group I. All these lines were resistant to the two races, a result which suggests that Garry possesses the Ajax gene or genes for resistance to the races in Group I.

The results in Table X for the Canuck × Victory cross differ somewhat from those in the other three crosses. Of the parents used in this cross, Victory is completely susceptible to all races, whereas in each of the other three crosses both parents were resistant to races 1, 2, 3, 5, 7, and 12. The lines that were classified as resistant in F_2 were either resistant to all races,

TABLE IX

SEEDLING REACTION TO 12 RACES OF STEM RUST OF F_3 LINES OF THE AJAX × GARRY CROSS, PREVIOUSLY CLASSIFIED IN F_2 AS RESISTANT AND SUSCEPTIBLE UNDER FIELD CONDITIONS

F_3 line No.	F_2 field reaction	F_3 seedling reaction to 12 races											
		1	2	3	5	7	12	4	6	8	10	11	13
93	R	R	R	R	R	R	R	R	R	R	R	R	R
101	R	R	R	R	R	R	R	R	R	R	R	R	R
117	R	R	R	R	R	R	R	R	R	R	R	R	R
118	R	R	R	R	R	R	R	R	R	R	R	R	R
124	R	R	R	R	R	R	R	R	R	R	R	R	R
89	R	R	R	R	R	R	R	Seg	Seg	Seg	Seg	Seg	Seg
91	R	R	R	R	R	R	R	Seg	Seg	Seg	Seg	Seg	Seg
92	R	R	R	R	R	R	R	Seg	Seg	Seg	Seg	Seg	Seg
93	R	R	R	R	R	R	R	Seg	Seg	Seg	Seg	Seg	Seg
144	S	R	R	R	R	R	R	S	S	S	S	S	S
145	S	R	R	R	R	R	R	S	S	S	S	S	S
149	S	R	R	R	R	R	R	S	S	S	S	S	S
152	S	R	R	R	R	R	R	S	S	S	S	S	S
154	S	R	R	R	R	R	R	S	S	S	S	S	S
151	S	R	R	R	R	R	R	Seg	Seg	Seg	Seg	Seg	Seg
172	S	R	R	R	R	R	R	Seg	Seg	Seg	Seg	Seg	Seg
174	S	R	R	R	R	R	R	Seg	Seg	Seg	Seg	Seg	Seg
181	S	R	R	R	R	R	R	Seg	Seg	Seg	Seg	Seg	Seg

or gave segregating reactions to all of them. All of the lines that were classified as susceptible in F_2 were susceptible to all of the races, except 1, 4, and 11. Lines 42, 80, and 140 gave an (R-) reaction to race 1, and all lines with the exception of 142 gave (R,R-), (R-) and segregating reactions to races 4 and 11. Line 142 was susceptible to race 4, but this reaction may have been due to too few seedlings having been inoculated. As previously mentioned the resistant reaction to these three races is due to an additional factor contributed by the Jostrain parent.

TABLE X

SEEDLING REACTION TO 12 RACES OF STEM RUST OF F_3 LINES OF THE CROSS CANUCK × VICTORY, PREVIOUSLY CLASSIFIED IN F_2 AS RESISTANT AND SUSCEPTIBLE UNDER FIELD CONDITIONS

F_3 line No.	F_2 field reaction	Physiologic races of stem rust											
		1	2	3	5	7	12	4	6	8	10	11	13
10	R	R	R	R	R	R	R	R	R	R	R	R	R
67	R	R	R	R	R	R	R	R	R	R	R	R	R
72	R	R	R	R	R	R	R	R	R	R	R	R	R
92	R	R	R	R	R	R	R	R	R	R	R	R	R
93	R	R	R	R	R	R	R	R	R	R	R	R	R
8	R	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg
87	R	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg
17	R	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg
43	R	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg
48	R	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg
52	R	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg
117	R	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg	Seg
34	S	S	S	S	S	S	S	R-,S	S	S	S	R-,S	S
42	S	R-	S	S	S	S	S	Seg	S	S	S	R,R-	S
80	S	R-	S	S	S	S	S	Seg	S	S	S	R,R-	S
140	S	R-	S	S	S	S	S	R-	S	S	S	R-	S
142	S	S	S	S	S	S	S	S	S	S	S	R-,S	S

From the plant breeding standpoint it is of interest to note that lines that are resistant to race 8 are, with few exceptions, resistant to all races.

Relation Between Seedling and Field Reaction

The F_3 of three crosses, Beacon × R.L. 1681, R.L. 1114.1 × R.L. 1681, and Ajax × Garry, were grown in the field with all 12 races present to compare the seedling reaction to race 8 with the field reaction to all races. The data are given in Tables XI, XII, and XIII. The agreement was good in all three crosses, especially in the Ajax × Garry cross, in which the inheritance was apparently governed by a single main gene. It is of interest to note that the (R-) class recorded in the seedling stage was also observed in the field, such plants being infected to the extent of from 5 to 10%.

As shown in Table XI, 80 lines of the Beacon × R.L. 1681 cross were grown in the field. There was complete agreement between the seedling and field reactions in the 27 lines of the segregating (R;R-;S) class, as well as in the eight susceptible lines tested. The agreement was less complete for the other four rust classes. Of 25 lines classed as resistant to race 8, 23 were resistant in the field, while two gave a segregating reaction. Of the seven lines classed as (R-) to this race, four produced the (R-) type of reaction, one produced resistant as well as (R-) plants, and two segregated producing resistant, (R-), and susceptible plants. Four lines that were classed as (R;R-) in the greenhouse gave a resistant reaction in the field; and of the nine lines of the

TABLE XI

RELATION BETWEEN F_3 SEEDLING REACTION TO RACE 8 AND F_3 FIELD REACTION TO ALL RACES IN THE CROSS BEACON × R.L. 1681

Seedling reaction to race 8	Field reaction to all races						Total
	R	R-	R;R-	R;R-;S	R-;S	S	
R	23	—	—	1	1	—	25
R-	—	4	1	2	—	—	7
R;R-	4	—	—	—	—	—	4
R;R-;S	—	—	—	27	—	—	27
R-;S	—	2	4	—	2	1	9
S	—	—	—	—	—	8	8
Total	27	6	5	30	3	9	80

segregating class (R-;S) two gave similar reactions in the field, two produced the (R-) type of reaction, four the (R;R-) type, and one gave a susceptible reaction.

One hundred and fifty-three lines of R.L. 1114.1 × R.L. 1681 cross were grown in the field and except for minor variations, the results shown in Table XII are in accord with those recorded in Table XI.

TABLE XII

RELATION BETWEEN F_3 SEEDLING REACTION TO RACE 8 AND F_3 FIELD REACTION TO ALL RACES IN THE CROSS R.L. 1114.1 × R.L. 1681

Seedling reaction to race 8	Field reaction to all races						Total
	R	R-	R;R-	R;R-;S	R-;S	S	
R	34	5*	4	3	—	—	46
R-	—	8	1	—	—	—	9
R;R-	2	—	10	1	—	—	13
R;R-;S	—	—	1	64	—	—	65
R-;S	—	—	—	—	14	1	15
S	—	—	—	—	—	5	5
Total	36	13	16	68	14	6	153

* *Three lines showed only a trace of rust.*

The field reactions of 173 lines of the cross Ajax × Garry agreed exceptionally well with those obtained in the greenhouse to race 8 as shown in Table XIII. A perfect agreement would have resulted except for the fact that out of 34 lines classed as resistant in the seedling stage two segregated in the field, with one of the lines producing only one susceptible plant. The homozygous (R-) and the segregating class (R-;S) were not observed in the field for this cross.

TABLE XIII

RELATION BETWEEN F_3 SEEDLING REACTION TO RACE 8 AND F_3 FIELD REACTION TO ALL RACES IN THE CROSS AJAX × GARRY

Seedling reaction to race 8	Field reaction			Total
	R	R;R-;S	S	
R	32	2	—	34
R;R-;S	—	91	—	91
S	—	—	48	48
Total	32	93	48	173

Discussion

The discovery that about 10% of the plants in the variety Hajira possessed resistance to all the currently known races of oat stem rust suggests a point of great importance in connection with the production of rust resistant varieties, not only in oats but in other crops as well. The genes governing rust resistance cannot be created at will by the investigator but must be searched for on the assumption that they exist somewhere in nature. In this search it should not be assumed that varieties, even if they are known to have originated as single plant selections, are necessarily uniform in rust reaction. Through natural crossing, or through mutation, genes for resistance to rust may arise in any variety. The discovery of the resistant plants in Hajira is no isolated occurrence but can be duplicated in other cereals and probably in many other types of plants as well.

Just as many noted "amateur" plant improvers have made a practice of examining varieties for morphological "off-types" which were later subjected to study, so should the rust investigator subject known varieties of plants to study on the chance that valuable genes for rust resistance may exist in them.

In the earliest work (8) reported by Welsh on crosses of other oat varieties with Hajira there was little or no opportunity of discovering the genes governing resistance to all races as the very races that would have facilitated the discovery, races 6 and 8, were excluded from the artificially induced field epidemic and were not used in greenhouse studies because it was assumed that all hybrid progeny were susceptible to them. Once this type of resistance was discovered, interest in its manner of inheritance was shown by oat breeders concerned with the production of rust resistant oat varieties.

Studies by Foote, quoted by Kehr and Hayes (4), indicated that the resistance to all races of stem rust possessed by selections from the Hajira–Joanette or Victoria–Hajira–Banner combinations was determined by a single factor. The results in the present paper with the exception of two crosses agree with the above. Two genes were found to govern resistance to all races in the R.L. 1114.1 × R.L. 1681 cross; and in Canuck × Victory it was

determined that Canuck received a gene for resistance to all races from the Hajira parent and a gene for resistance to race 11 from the Jostrain parent. Probably, this gene contributes resistance to races 1 and 4 as well since the reactions to these two races (Table X) were the same as those to race 11. Kehr and Hayes (4) using the Hajira–Joanette type of resistance in tests with race 6 found that more than a single gene was involved in inheritance.

Lines that were resistant to race 8 in the seedling stage were found to be resistant to the other 11 races, though in the case of race 6 this resistance is to a considerable extent influenced by temperature. Furthermore, there was a very close agreement between the seedling reactions to race 8 and the field reactions to all races. These results show that, in the Hajira type of resistance, resistance to race 8 is indicative of resistance to all races.

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AEROBIOLOGICAL SAMPLING METHODS FROM AIRCRAFT[1]

By C. D. Kelly, S. M. Pady, and Nicholas Polunin

Abstract

Three methods are described for sampling airborne bacteria and fungi from aircraft. These methods were designed for use in aircraft flying at speeds about 200 m.p.h. The first is a qualitative method for use in a B-29 aircraft, with a sampling tube to hold a Petri dish 12 in. in front of the nose, yet permit loading and unloading from the interior. In the second and third methods, which are quantitative, the samplers are located inside the aircraft and air is brought to them by rubber hose connected to 1 in. aluminum elbows in a side window; the sampled air leaves the aircraft by another set of hose and elbows. The second sampling method uses a G.E. Electrostatic Bacterial Air Sampler located in an aluminum sampling chamber 18 in. × 18 in. × 18 in. The third method utilizes filters made from glass wool and lens paper discs mounted in brass hose couplings. Numbers of organisms were calculated on a cubic foot basis from colonies which developed on nutrient media. In the second method, the organisms were precipitated directly on the agar surface; in the third method, however, the filters were shaken in sterile water and aliquot portions cultured in agar plates. For studying the numbers of fungous spores, silicone slides were substituted for agar in Petri plates exposed in the G.E. sampler. Typical results are given for the two quantitative methods.

Introduction

Ever since the days of Pasteur, there have been attempts to determine the numbers and kinds of microorganisms in the air. Pasteur demonstrated the presence of microorganisms in the atmosphere by drawing air into a flask containing sterile broth; later he used guncotton as a filter to remove air-borne bacteria. Most of the methods used by subsequent investigators were developed to study specific problems and were satisfactory for the purpose for which they were designed. This was particularly true where the air being tested was indoors, the number of organisms high, and the sampler stationary.

The methods employed in sampling air-borne microorganisms fall into two main categories, qualitative and quantitative. In the former, Petri dishes (agar plates) or sticky slides are exposed to the air; in the latter various devices are employed to remove all of the organisms from a known volume of air. The apparatus that has been used in the quantitative methods may be divided into three main types; (*a*) impingers (Owen's dust impinger, Wells' air centrifuge); (*b*) aspirators (Rettger's air washer); (*c*) filters (Pasteur's air filter, Petri's sand filter, Proctor's filter magazine). A National Research Council (U.S.) Committee on aerobiological apparatus has adequately discussed these types in a review (2). However, mention should be made of two recently developed samplers not covered in the above review (2): the Slit Sampler (1) and the General Electric Electrostatic Bacterial Air Sampler (3, 4). The Slit Sampler employs a vacuum pump to draw air through a slit at the rate of 1 cu. ft. per min. The organisms impinge upon the surface

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of a revolving agar plate 2 mm. below the slit. The General Electric Sampler was designed to draw air at 1 cu. ft. per min. over two Petri plates between strong electrostatic fields thereby precipitating the organisms onto the surface of the agar.

For outdoor air, and particularly when sampling is done from aircraft, a special technique is required. This is due in part to mechanical difficulties, and in part to the much lower number of microorganisms in the outdoor air. The sampling methods reported in the literature for use in aircraft are chiefly qualitative, Petri plates being held by hand in the air stream or a holder being used for slides. The only quantitative apparatus designed for use in aircraft was developed by Proctor (9) for sampling air over Boston.

This paper constitutes a report on qualitative and quantitative methods, particularly the latter, which were developed in connection with studies of arctic air made from aircraft with moderately high air speeds.

Qualitative Methods

In the summer of 1947, agar plates and vaselined slides were exposed in the Canadian Arctic and Subarctic during a series of flights with the R.C.A.F. (8). The slides were placed in a holder and held in the airstream for five minutes; during this time, the plates were exposed by hand for two minutes (6, 7).

During 1948, an opportunity presented itself through the courtesy of the U.S.A.F. to take samples between Fairbanks and the North Pole from a B-29 aircraft. Because of the speed of the plane, low temperatures, and possible pressurization of the cabin, a piece of apparatus was designed (Fig. 1, Sampler No. 1) to expose a standard Petri dish in front of the nose of the plane yet to permit loading and unloading of the Petri dishes inside the aircraft. A steel plate was fitted in place of the main front plexiglass bombardier's window and a steel tube (Fig. 1B) about 12 in. long and 4 in. inside diameter was welded in a horizontal position to cover an opening in the plate. A second steel tube $3\frac{7}{8}$ in. outside diameter, 27 in. long, plugged at both ends, was fitted inside the first tube so that it would slide easily in and out (Fig. 1C). Spring steel clips were made to fit the sides of a Petri dish and hold it in place at the front end of the second tube. A breech (Fig. 1E) with a cover (Fig. 1F) was cut in the side of the first tube to allow loading and unloading of the Petri dishes when the second tube was withdrawn. A slide shutter (Fig. 1D) was fitted to cover the hole in the steel plate when the tube was not in use.

The tube was withdrawn and shutter closed in the nonsampling position. Loading consisted of opening the breech cover, placing a sterile covered Petri dish into the opening, and forcing the dish into position in the holder, then the lid was removed and breech cover closed. To make the exposure, the shutter was opened and the inner tube extended so as to place the Petri dish 12 in. in front of the nose. At the end of the sampling period the process was reversed.

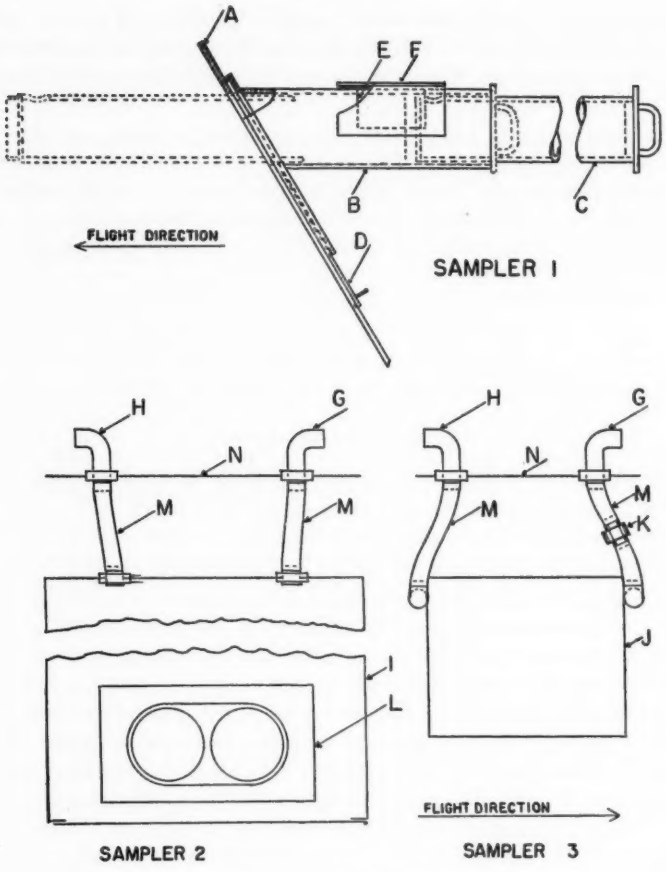


FIG. 1.—Diagrams of the three samplers.

A. Steel plate in place of plexiglass window.
B. Outside steel tube, or sleeve.
C. Inside steel tube with spring clips at end to hold Petri dish.
D. Slide shutter.
E. Breech.
F. Breech cover.
G. Inlet aluminum elbows.
H. Outlet aluminum elbows.
I. Sampling chamber.
J. Flow meter.
K. Filter.
L. General Electric Sampler.
M. Rubber hose.
N. Plexiglass window of escape hatch.

Quantitative Methods

In devising equipment for quantitative sampling from aircraft certain factors were kept in mind. The apparatus should be simple, efficient, light, easily portable, and as nearly self-contained as possible, so that it could be used in the cabin of any type of aircraft. The two types of apparatus described here was made with these requirements in mind. One utilized a special sampling chamber containing constantly changing outside air, in which a General Electric Electrostatic Bacterial Air Sampler was mounted. This apparatus was called the McGill G.E. Sampler and will be described below as Sampler No. 2. The other employed a glass wool filter system and is referred to as Sampler No. 3.

Outside air was brought to the samplers by movement of the plane, thus eliminating the need for a pump. Aluminum elbows of 1 in. diameter were fitted into the plexiglass side window of the plane to act as inlets and outlets, the former facing forward and the latter aft, an arrangement which creates a "venturi" effect. This device has been used mostly in "North Star" and "C-54" aircraft, and, by having the inlets and outlets installed in a spare "escape hatch" door, which is interchangeable in both types of plane, it is possible to install the equipment in a few minutes.

Sampler No. 2

The G.E. sampler was designed to operate in a room with gentle air flow and it was therefore necessary when using it in a fast moving plane to create a sampling chamber in which there would be a constantly changing slow flow of outside air. The sampling chamber was an aluminum box 18 in. × 18 in. × 18 in. in dimension (Fig. 1I) connected to the inlet (Fig. 1G) and outlet (Fig. 1H) by 1 in. rubber tubes 2 ft. – 3 ft. long (Fig. 1M). The inlet hose delivered the air into the upper left-hand corner of the sampling chamber while the air made its exit by an opening at the lower right, immediately opposite the exit of the G.E. Sampler (Fig. 1L). As the sampler was designed to run on 110 volts a-c. current, it was necessary to install a converter in order to use the 24 volts d-c. current of the plane. Plate I shows details of Sampler No. 2 when assembled.

In operation, the rubber tubes and sampling chamber were scoured out by allowing an unrestricted full flow of air. This scouring was carried out for 15 to 20 min. immediately upon take-off. The procedure in taking the samples was as follows: the air flow was reduced to 5 cu. ft. per min. by means of clamps on the hoses, the chamber door was opened, the lucite lid of the G.E. sampler was raised, Petri plates were placed in position, the lid and door closed, and the current turned on for the required period. A positive pressure was maintained so that when the door was opened, air from the inside of the aircraft did not enter the sampling chamber.

The advantage of the McGill G.E. method is that bacteria and fungi are precipitated directly on the plates and, after five days' incubation at 25° C., the number of colonies can be determined. A further advantage is that

silicone plates and slides could be used (5). The chief disadvantage of the McGill G.E. method is that it is restricted to sampling 1 cu. ft. of air per minute. In air with a low number of microorganisms, this necessitates sampling for long periods, reducing greatly the number of samples that may be obtained.

Sampler No. 3

It was decided to design a filter, since filters are efficient in removing organisms from the air and also permit a large volume of air to be sampled. A further fact, and one of great importance, is that the filter permits suitable dilution and the use of a variety of media. Clumps of fungous spores or clusters of bacteria on a dust particle would also be broken up by this method, thus giving a more accurate determination of the numbers. There is a further advantage in that the filters may be stored for a period of time after exposure. After many tests, a satisfactory filter was made by using a brass hose coupling (Fig. 1K) (1 in. inside diameter) with glass wool and lens paper discs (Plate II) acting as filtering agents.

In preparing the filters, discs were cut with a $1\frac{1}{8}$ in. cork borer from glass wool as it comes from a roll and set in the brass hose coupling in place of the usual rubber washer. Because there was a tendency for the fibers of the glass to shear in use, discs of lens paper were placed between and at the outside of the glass wool discs. The complete filter contained 16 discs of glass wool and five discs of lens paper: the ends were plugged with cotton, then covered with parchment paper caps (Plate II, B, C). Each filter was wrapped in a paper towel, steam sterilized, and dried at 45° C. for 72 hr. to remove any moisture (Plate II, D).

The inlet and rubber tubing were scoured by free flowing air for 15-20 min. immediately after take-off. The air was then shut off by means of clamps and the filter connected to the inlet, flow meter, and outlet (Fig. 1K). In operation, after removal of the clamps, the air flow varied from 1.5 to 3 cu. ft. per min. in the R.C.A.F. "North Star", and, as a rule, the length of exposure was one hour or longer. In the laboratory, the discs were removed with sterile forceps, placed in 50 ml. of sterile water in a sterile flask and shaken for 10 min. Aliquots of 2 and 5 ml. were placed in sterile Petri dishes and agar added. Incubation was at 25° C. for five days.

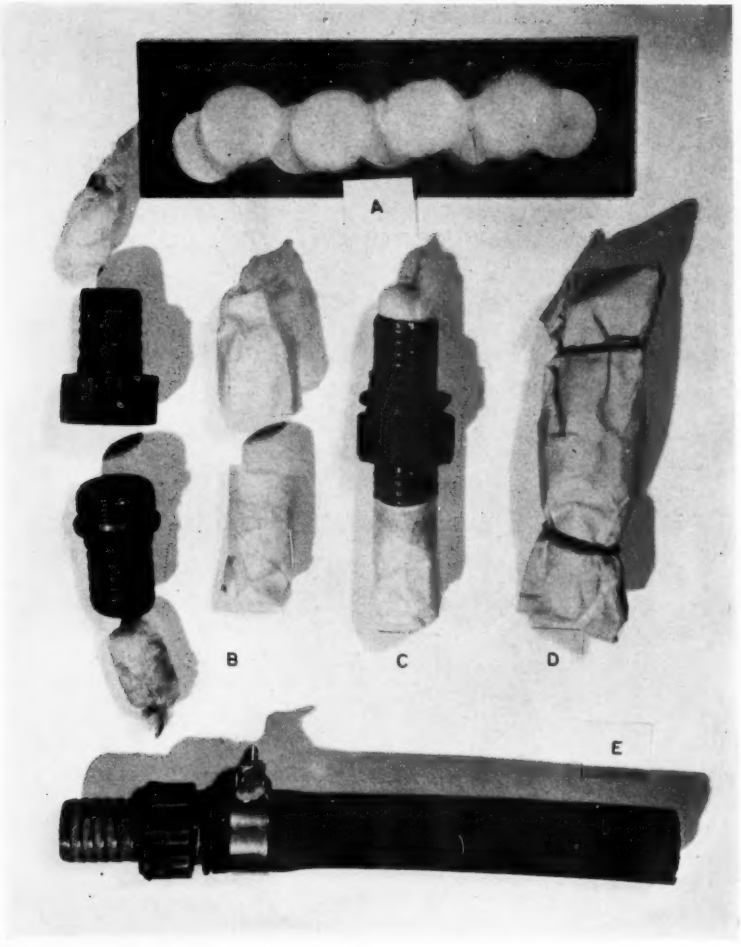
Sampling Results

Typical figures obtained by the two quantitative methods are illustrated by the results of a local test flight made on June 21, 1949 (Table I).* The flight was made in unsettled weather which followed 10 days of clear warm weather with tropical southerly winds. The first part of the flight was in tropical air, but in the latter part of the flight, an arctic air mass was encountered. Exposures were made in both air masses using filters as well as agar plates and silicone slides. Four parallel sets of exposures were made (Tests 1-4)

* *Data for Sampler No. 1 are being published separately.*

PLATE I

Photographs of the McGill G.E. sampler: left, side view with escape hatch and flowmeter; center, front view showing sampling chamber, lucite door, stand and converter; right, sampling chamber open to show McGill G.E. sampler.



Filter.

A. Glass wool and lens paper discs.
B. Parts of filter holder and parchment caps.
C. Filter assembled showing paper cap on one end and cotton plug in other.
D. Complete filter wrapped in paper towel.
E. Filter showing rubber hose fastened at one end.

TABLE I

DATA FROM FLIGHT OVER MONTREAL, JUNE 21, 1949

Test No.	Method*	Exp. length, min.	Total cu. ft.	Organisms per cu. ft.**			Weather	Air*** mass	Wind direction	Alt., ft.	Air speed, knots
				Bacteria	Fungi	Total					
1	F	30	48.2	0.8	15.8	16.6	Cloudy Rain	mT	220°	4900*	190
	GEP	15	7.5	0.1	22.0	22.1	Rain	mT	220°	4900	190
	GEP	15	7.5	0.8	25.3	26.1	Rain	mT	220°	4200	190
	C	—	—	—	—	No colonies	—	—	—	—	—
2	F	30	46.9	1.4	10.2	11.6	Cloudy	mT	220°	5000	190
	GES	30	15.0	—	180.2	—	Cloudy	mT	220°	5000	190
3	F	30	44.8	0.5	8.9	9.4	Cloudy to clear	mT	220°	2500	190
								and	—	1700	190
								cP	315°	5000	190
	GEP	15	7.5	0.6	16.8	17.4	Cloudy to clear	mT	220°	2500	190
	GEP	15	7.5	0.5	9.1	9.6	Cloudy to clear	cP	315°	1700	190
										5000	190
	C	—	—	—	—	Two colonies	—	—	—	—	—
4	F	18	32.1	0.8	2.2	3.0	Cloudy	cP	215°	5000	190
	GES	18	9.0	—	4.2	—	Cloudy	—	215°	5000	190

** F = filters; GEP = agar plates from McGill G.E. Sampler; GES = silicone slides from McGill G.E. sampler; C = control.*

*** Numbers refer to colonies on agar for F and GEP, but direct counts of individual spores for GES.*

**** mT = maritime tropic; cP = continental polar.*

with the filters and McGill G.E. sampler using agar plates and silicone slides in alternate tests. Controls were run on the exposing and plating procedure. The medium was Czapek's solution agar with 0.1% yeast extract and 0.5% glucose.

It was surprising to find that the numbers of bacteria were essentially the same in both tropical (mT) and polar(cP) air masses whereas the fungi showed striking differences in numbers. There was a tendency for the bacterial colonies to be obscured or suppressed by overgrowth of fungi in the plates where the number of fungous colonies was high as in Test 1 (Table I). In maritime tropic air (Tests 1 and 3) the average number of fungal colonies obtained by the McGill G.E. sampler was 21.3 per cu. ft. while the bacteria averaged 0.5 per cu. ft. When the filters sampled the same air mass (Tests 1 and 2), the counts averaged 13.0 and 1.1 for the fungi and bacteria respectively. Test 4, in continental polar air taken with a filter, gave counts of 2.2 for fungi and 0.8 for bacteria. Where the air was mixed (Test 3) the numbers were intermediate.

The readings from the silicone slides gave very interesting results (Table I). The number of fungous spores per cu. ft. was obtained by counting an area covered by a cover glass 22 × 50 mm. and corrected for the area of the Petri dish. The number of individual spores was found to be many times greater than the corresponding number of colonies on agar plates. In Test 2, in which the slide and filter were exposed simultaneously, the number of spores was 180.2 per cu. ft. while the number of colonies was 10.2 per cu. ft. In Test 4, the number of spores was double the number of fungous colonies obtained from the filter. Comparison of the slide from Test 2 with the agar plates exposed in Test 1 also indicate much higher numbers.

Discussion

While any sampling apparatus has certain inherent limitations, the two quantitative types used in this work, namely the McGill G.E. sampler and the filter sampler, seemed satisfactory for sampling under the conditions for which they were designed. In our studies in arctic air, where the number of organisms was very low, the samplers worked satisfactorily although a long period of sampling was required. Despite this limitation, the quantitative methods are so superior to any qualitative method that the use of the latter does not appear to be justified except under special circumstances.

The efficiency of the General Electric sampler has been established by Luckiesh *et al.* (3). On agar plates exposed in this sampler, colonies develop that are easily counted, for the organisms are precipitated directly onto the agar. This simplifies the work of culturing both the bacteria and fungi as they can be isolated directly from the plates after incubation and counting. The volume of air sampled is 1 cu. ft. per minute so in order to obtain satisfactory plates a long period of exposure may be necessary which would, in the case of an hour-long sample, cover a distance of over two hundred miles,

yet sample only 60 cu. ft. of air. Because the organisms are deposited and grown on the surface of the agar, there is always the possibility of a colony having grown from a clump of cells or spores and therefore not giving a true estimate of the actual number of microorganisms in the air. Also in many cases, particularly with the bacteria, the organisms are carried on dust particles. A particle may carry a single cell, a group of cells of the same species, or cells of two or more species which give a mixed colony. This nonseparation of groups of spores or bacterial cells would appear to be the only serious criticism of the G.E. sampler.

The third sampler, the filter, is also efficient in removing organisms from the air and has the added advantage of sampling larger volumes of air. Moreover, clumps of cells tend to be separated during shaking and those adhering to dust particles are washed off, which reduces the incidence of mixed colonies. With proper dilutions, more accurate counts are obtained as most of the colonies are subsurface ones; this tends to prevent overgrowth of the bacterial colonies by the fungi. While the results of the filter sampler as compared with those of the McGill G.E. sampler are in close agreement in so far as the bacteria are concerned, the counts for the fungi are much lower. This suggests either that not all of the spores were held by the filter or that some of the fungous spores remained in the filter when dilutions are made. Another problem is that the large amount of manipulation necessary for culturing the organisms from the glass wool increases the possibilities of contamination. The filter method thus requires considerably more time and shows a much lower efficiency at least with respect to the fungi than that in which the McGill G.E. sampler is used.

One very surprising result was that the number of fungous spores obtained on the slides was much greater than the corresponding number of colonies which grew on the nutrient plates. It is our opinion that this difference is due primarily to loss of viability of fungous spores during air transit. The viability of air-borne spores may be directly related to the length of time that they remain in the air. Moreover, spores of rusts and also of many smuts will not germinate and grow on artificial media. If many spores of rusts and smuts were present on the slides, it would be expected that the colony count on nutrient agar would be reduced by that amount. Two other factors may also enter into the picture at this point, namely, the medium might be unfavorable for certain fungi, and also that such spores as are in clusters or groups make only one colony on the G.E. plates, yet may be counted as individual spores on the slides. It is considered, however, that the chief cause of the lower counts on the agar plates is loss of viability. In other words, the majority of the fungous spores on the slides are no longer capable of growth.

The numbers of bacteria found in both tropical and arctic air were lower than would seem to be indicated by the results of 1947 (6) and in the tests reported here, with one exception, were less than one per cubic foot. One must assume either that the numbers of bacteria are very low in both air masses or that the development of bacterial colonies was inhibited by

fungous colonies. In support of the latter assumption it may be pointed out that the count of fungi on plates and slides indicates the tropical air to be carrying an extremely heavy load of fungous spores. Since most of the microorganisms in the air are of soil origin (9, 10), it might be expected that the presence of high numbers of fungi would indicate correspondingly high numbers of bacteria. It is therefore possible that the development of bacterial colonies has been suppressed and the actual numbers are higher than our figures indicate.

It is evident that the geographical source of the prevailing wind is of paramount importance in any aerobiological investigation. It seems that southerly winds carry large numbers of organisms northward as indicated in the results from the flight reported in this paper. Prior to the flight of June 21, 1949, the wind had been from the south and was laden with fungous spores and bacteria arising possibly even as far south as the tropics. Parts of the flight were made in warm southern air while the balance was in cool polar air, affording a contrast in the numbers of microorganisms carried by the two air masses. More data are needed, however, before any conclusions can be drawn regarding average loads carried by air currents, but there are clear indications of a close correlation between specific air masses and the numbers of bacteria and fungi contained therein. The most important factor in aerobiological sampling would appear to be the origin, direction, and duration of the air which is being sampled.

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A HISTOLOGICAL STUDY OF SUGAR MAPLE DECAYED BY *POLYPORUS GLOMERATUS* PECK[1]

By H. M. Good[2] and C. D. Nelson[3]

Abstract

Parenchyma cells of maple wood were found to remain alive for many years after incorporation into the heartwood. In wood invaded by *Polyporus glomeratus* Peck these cells had been killed in advance of the spreading mycelium, and their contents transformed into masses of dark brown wound gum. These, and similar masses which occasionally formed in the vessels and fibers, gave the outer part of the infected heartwood a characteristic dark color. The presence of wound gum appeared to inhibit development of decay, possibly by reason of the increase in pH with which it has been associated consistently. In tissues containing wound gum, decay was limited to slight delignification of certain cells in the intervessel areas, but following its disappearance disintegration of the tissues was rapid. Variation in resistance to decay was found to be related to variations in the reactions of cell walls to several staining procedures.

Introduction

There is a high incidence of heartrot in sugar maple in Ontario. Nordin (11, 12) has found that 85% of trees studied were decayed to varying extents. The trees examined were predominantly over 5 in. in diameter, measured at breast height outside the bark. The fungus occurring most frequently, and causing considerable cull in merchantable trees, was *Polyporus glomeratus* Peck, previously reported on maple by a number of authors (2, 13).

An investigation of maple trees 3 to 6 in. in diameter, and from 30 to 60 years old, at the Queen's Biological Station,* has shown that these younger trees are also seriously affected by heartrot fungi. Eighty percent of trees in three sample blocks had open rot lesions, and subsequent studies have shown that few, if any, of the remainder are completely free from decay. *P. glomeratus* proved to be the cause of decay in 14 of the 50 trees from which isolates have been obtained.

In view of the importance of *P. glomeratus* on maple in Ontario, a study of infected trees has been made to provide information on spread of the fungus in the tree and the nature of its parasitism. Certain properties of normal wood which appeared related to the development of decay, the distribution of the fungus in the diseased tissue, and the effects of the fungus upon the wood, have been considered.

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Contribution from the Department of Biology, Queen's University, Kingston, Ont. This study was carried out with the assistance of grants from the National Research Council and the Research Council of Ontario.

[2] *Assistant Professor.*

[3] *Student Assistant.*

* *Queen's University Biological Station is situated near Chaffey's Locks, Ont., about halfway between Kingston and Ottawa on the Rideau Canal.*

Materials and Methods

Material was obtained from living trees near the Biological Station Laboratory. For the most part, wood was brought into the laboratory and sectioned immediately on a sliding microtome. Fresh wood cut well, and sections as thin as 10 μ could be obtained without difficulty. In general, radial sections were used for study of the fungus in the wood, and transverse sections for changes in the wood structure due to fungus activity. As some of the material could not be examined during the summer it was air-dried, stored, and boiled before sectioning.

Sections for the study of fungus in the wood were stained with picro-aniline blue and safranin after the method of Cartwright (3). Dilute neutral red chloride, 5 p.p.m. was used as a vital stain. For study of wood breakdown, sections were treated with various reagents which give more or less specific tests for constituents of woody cells (14).

Fungi causing the rots were isolated by placing chips of diseased wood on 2% malt extract agar slants and identifying the organism growing out of the wood either by cultural characteristics (10) or by direct comparison with cultures of maple rot fungi obtained from the collection in the Division of Botany and Plant Pathology, Ottawa. Material from eight trees decayed by *P. glomeratus* was examined, together with normal wood from numerous additional trees.

The gross features of *P. glomeratus* rot of maple have been described (2). The fungus occurs principally in the heartwood, with rare areas of spread into the sapwood. It produces a light spongy rot surrounded by a considerable area of firm dark wood (Fig. 1). In the following discussions reference will be made to three regions of the diseased trunk, normal, dark brown, and center. The term center is applied to all of the light spongy rot characteristic of *P. glomeratus* decay.

Observations on Normal Maple Wood

Longevity of Cells

It has been stated that parenchyma cells of sapwood are alive, and that, in the change to heartwood, food reserves are withdrawn and the cells die (4). This view seems questionable in view of the contributions of MacDougal (8) and White (16), who have presented evidence that cells may live for some time after being incorporated into heartwood. A definition of heartwood to exclude living cells would not resolve the problem, since too little is known of the longevity of woody cells for such a definition to have significance. The term heartwood should therefore be used in its traditional sense, to designate the central, darker, part of the trunk. At present no generalization can be made about the presence of living cells in this tissue.

Whether cells in heartwood are alive is an important question in studies of decay of living trees. The presence of living cells, even in small numbers, would introduce the possibility of relations between these and an invading

fungus quite distinct from the relations of the fungus to an inert substrate. Maple heartwood was investigated, therefore, to determine whether it contained living cells. Cells were considered to be alive if they accumulated a vital stain, and could be plasmolyzed and deplasmolyzed.

Radial sections 30 μ thick were prepared from wood of freshly cut trees. These sections proved thin enough for microscopic study and thick enough to ensure that many of the cells would be uninjured. Immediately after sectioning they were placed in a solution of neutral red chloride, and after varying periods of time were mounted in the same solution and examined under the microscope. The first indication of an accumulation of the stain was seen after two hours' immersion, by which time the phloem ray cells had become quite red. At this time no stain had appeared in the adjacent xylem cells. However, after four hours there was a trace of color in the xylem ray cells, and in five hours these were stained distinctly. Staining continued to improve, and best results were obtained with sections which had remained in the solution overnight.

Following the uptake of neutral red, the sections were placed in a 0.6 *M* solution of sodium and calcium chlorides and observed under the microscope. The cells which had become stained became plasmolyzed (Fig. 5), and on the addition of water regained their original condition (Fig. 6). Plasmolysis and deplasmolysis were carried out several times. Ray cells proved surprisingly hardy and have been plasmolyzed after five days' immersion in the neutral red solution.

In sound 50-year-old trees, showing about 15 annual rings of sapwood, the ray cells were alive to the center. In decayed trees live cells were found as far in as the edge of the brown zone, a few living cells occurring mixed with the dark cells. Many cells were cut open in sectioning, but rays in the center of the sections were uniformly stained and all the cells could be plasmolyzed. In the innermost rings of uninfected trees a few cells appeared which contained yellow granular masses. These cells did not take up the neutral red, nor plasmolyze, and were presumably dead. It may be concluded that practically all the ray parenchyma of maple wood remains alive for more than 40 years under the environmental conditions obtaining for this group of trees. The maximum life is undoubtedly much longer than this. As might be expected, the fibers appeared empty and dead. Living xylem parenchyma cells over 40 years old were seen, but did not constitute an appreciable part of the wood. On the other hand, rays have been estimated to make up at least 10% of the volume.

Distribution of Reserve Food

Since reserve food in invaded tissues becomes available to the pathogen, the distribution of stored food in maple wood was investigated. In sections of fresh wood mounted in Sudan III fat droplets were seen in practically all normal parenchyma cells. Seasonal fluctuations have not been checked, but during the summer a considerable number of globules occurred in cells of the

current seasons growth, and a reduced but fairly constant number in all other uninfected wood. While the fat globules were easily seen under the microscope they made up a very minor part of the wood. Preliminary tests of ether extraction of dried sawdust showed only a trace of ether-soluble material. Protein reserves appeared to occur in even smaller quantities than fats. Sections treated with Millon's reagent showed no indication of storage protein.

The most important reserve foods appeared to be starch and soluble sugars. In radial sections mounted in an aqueous solution of iodine and potassium iodide, starch appeared in all normal ray and xylem parenchyma cells (Fig. 2), except in the heart of a 45-year-old tree examined in November, in which some cells were alive and devoid of starch. Parenchyma cells in the sapwood were more densely packed with starch grains than were those in the heartwood. A few starch bearing cells were found among those with dark brown contents in the outer part of the diseased tissue, but these appeared to be still living. It is concluded that parenchyma cells of heartwood store starch, the amount varying with the age of the tissue and the season, and that most of the starch disappears before death of the cells so that relatively little occurs in the tissues invaded by *P. glomeratus*. However, an appreciable amount of sugar was found in the diseased wood. Paper chromatograms showed sucrose, fructose, and some glucose.

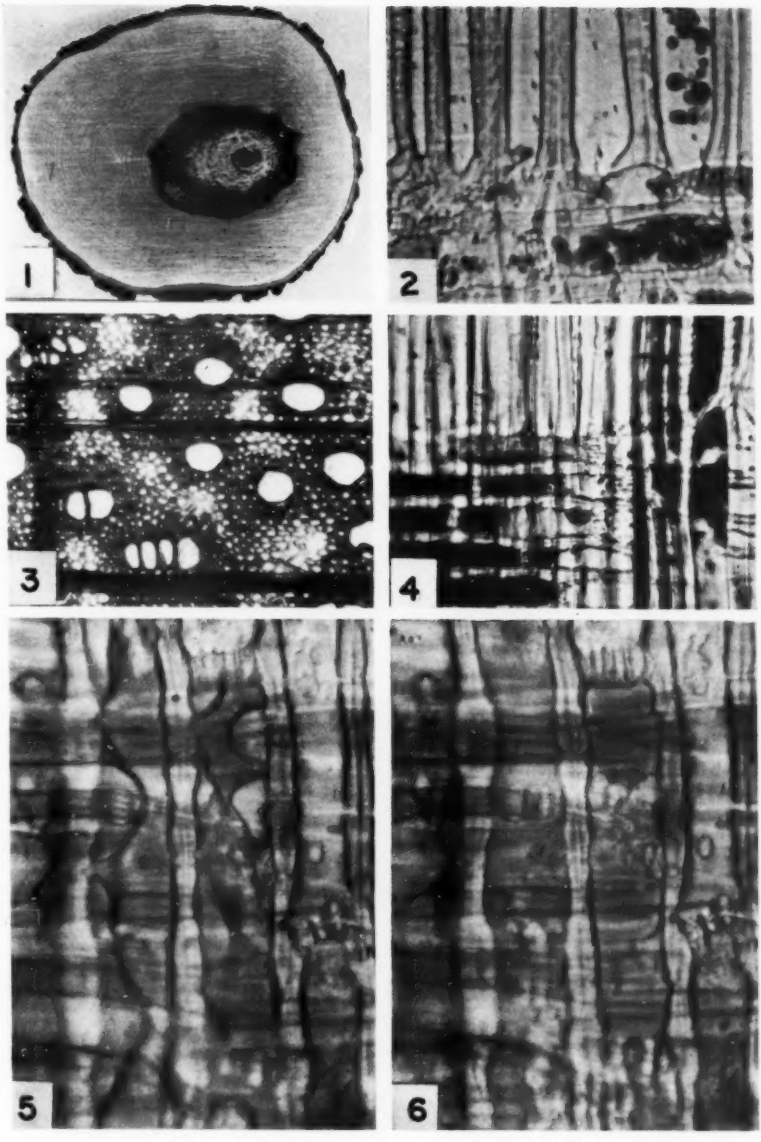
The work of Jones and Bradlee (7) on carbohydrate content of maple trees agrees with these observations and furnishes further evidence that the parenchyma cells are alive and active for long periods of time. These authors found a seasonal variation in the starch and sugar content of the inner wood of maple trunks. Starch was highest in summer and lowest in winter, sugars varying inversely. These changes can hardly be interpreted as functions of dead cells.

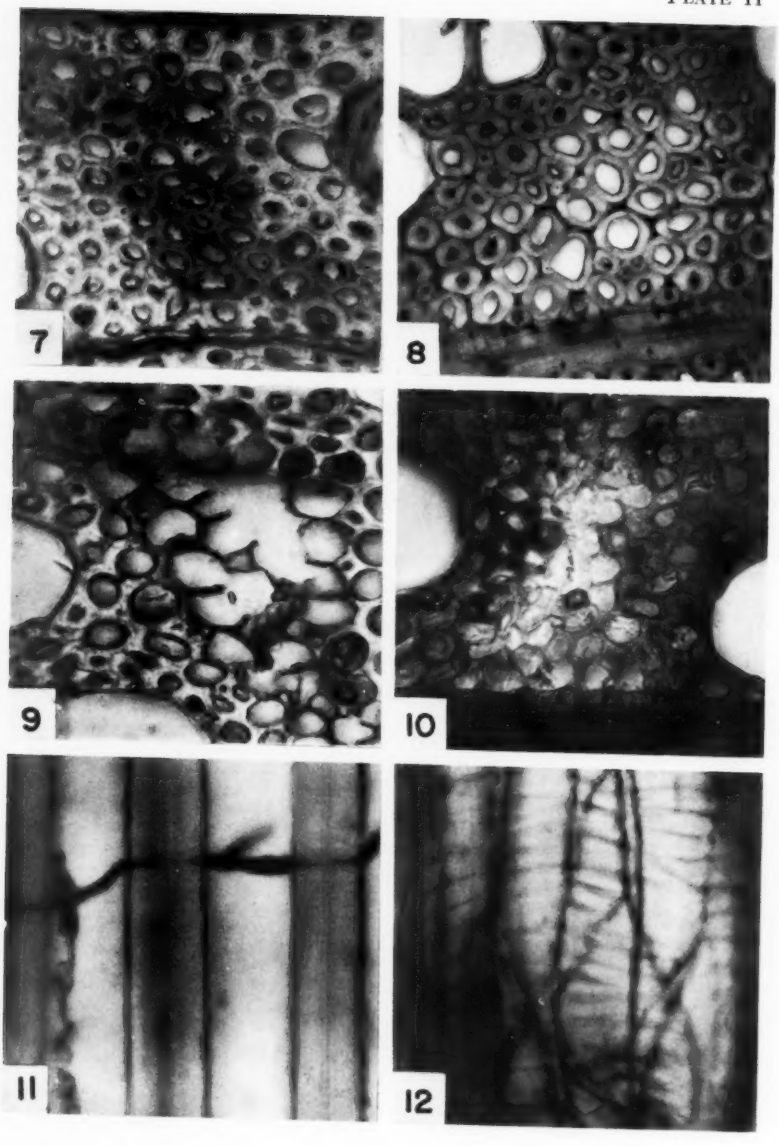
Variation in the Composition of Cell Walls

The observation that *P. glomeratus* digested certain elements of the wood much more rapidly than others prompted an examination of the form and composition of the cells of maple wood. The general arrangement of tissues in maple has been described (1) and will not be considered here except for brief reference to certain features of the cross section. The wood is diffuse porous with cells formed in the spring only slightly larger than those formed in summer. At the outer limit of the annual ring are several rows of small, very thick-walled fibers. Cells immediately around the vessels are smaller than those at a distance, and in the intervessel areas are groups of cells which are quite large with relatively thin walls (Fig. 8).

FIG. 1. *Polyporus glomeratus* decay in maple. × ½.
FIG. 2. Starch in 50-year-old ray and xylem parenchyma. × 600.
FIG. 3. Cross section of dark zone showing regional breakdown. Maule stain. × 125.
FIG. 4. Wound gum deposits in ray and xylem parenchyma. × 400.
FIG. 5. Thirty-six-year-old ray cells plasmolyzed. × 1200.
FIG. 6. The same cells as in Fig. 5 a few minutes after the addition of water. × 1200.

PLATE I

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Although precise microchemical tests for substances in the cell wall are not available, some information on wall composition may be obtained from the use of stains, particularly those which are somewhat specific for a certain type of substance. The phloroglucin–hydrochloric acid, and Maule tests for lignin, and the ruthenium red stain frequently used for pectic materials were therefore applied to transverse sections of normal and diseased maple wood.

Sections stained with phloroglucin–hydrochloric acid and with the Maule stain had essentially the same appearance both giving distinct lignin reactions. Scarth *et al.* (15) have concluded that such tests are roughly quantitative, the intensity of color being proportional to the lignin content. In the sections treated the middle lamellae* stained very deeply indicating a high concentration of lignin. The secondary walls also gave lignin reactions, but less than the middle lamellae. Moreover, the depth of color in the secondary walls varied considerably. On the basis of the intensity of stain the cells could be arranged in the following order of decreasing lignification; late summer fibers, vessels and adjacent cells, ray parenchyma, and intervessel fibers. These last were the large cells referred to above. Their reduced reaction to the Maule stain is shown in Fig. 8.

Sections stained in dilute ruthenium red also showed a marked variation in intensity of stain in the various types of cells. In general, this was in reciprocal relation to the intensity of the lignin tests, the larger fibers of the intervessel areas taking up the most stain (Fig. 7). Staining of the middle lamellae was somewhat variable. In some sections they were generally stained, in others only in occasional cells.

Ruthenium red has been used extensively as a stain for pectic materials, though its affinity for treated lignin has also been utilized (14). Sections were therefore subjected to the following treatments to remove pectic material; 2% ammonium oxalate for one-half hour, 5% ammonium oxalate for one hour, 2% ammonia for one-half hour, and 2% hydrochloric acid for one-half hour followed by 2% ammonia for one-half hour. A temperature of 90°-95° C. was maintained in all these treatments. The treated sections were washed thoroughly and stained with dilute ruthenium red. None of the treatments

** Middle lamella is used here to denote the distinct central line between cells. It probably includes most, if not all, of the primary walls in addition to the true middle lamella.*

FIG. 7. Cross section of the dark zone showing regional variation in intensity of ruthenium red stain. × 300.

FIG. 8. Cross section of normal heartwood showing a regional variation in intensity of Maule stain. × 300.

FIG. 9. Cross section of center area showing the strong ruthenium red stain in the wall residues. × 300.

FIG. 10. Cross section of center area, showing delignification and disintegration of one area such as shown in Fig. 8. Maule stain. × 300.

FIG. 11. Radial section of diseased wood showing the fungus in the fibers and penetrating the walls. Cartwright stain. × 2100.

FIG. 12. Radial section of diseased wood showing fungus in a vessel. Cartwright stain. × 1000.

removed the material taking up the stain though the last seemed to reduce the color in the secondary wall somewhat. On the other hand, all the treatments gave a marked increase in the color of the middle lamellae which stained brilliantly after treatment with hydrochloric acid or ammonia, and somewhat less brilliantly, but very distinctly, after treatment with oxalate. Apparently the differences in staining with ruthenium red are not due to variations in pectic content of the cells. From the effect of the treatments on the highly lignified middle lamella it would seem more probable that the lignin may be modified to a form which takes up the dye. If this is the case lignin must be presumed to occur in different forms in maple wood, the proportions of these forms varying in the different types of cells.

Studies on Decayed Wood

Distribution of Fungus in the Wood

The distribution of mycelium of *P. glomeratus* was checked both microscopically and by isolations. The fungus was isolated readily from practically all tissues showing signs of decay, but apparently did not occur in the outermost part of the dark brown zone, or in the regions above the typical decay, where discoloration was the only indication of infection. This distribution has been borne out by microscopic examination of sections of diseased wood. In all cases the discoloration extended a short distance beyond the area in which mycelium was found.

The fungus occurred in both fibers and vessels (Figs. 11 and 12) but was not found to spread through the rays. The hyphae were branched with occasional simple septa. Most were from 1.5 to 3.5 μ in diameter but some were as small as 0.5 μ. All had a granular appearance. Occasional pigmented hyphae were seen in the dark outer zone and in the central area where the tissues were very rotten. In most cases, however, the hypae were hyaline.

Hyphae passed freely through walls and were constricted in passage (Fig. 11). There was no sign of bore holes except in very rotten wood where considerable areas around the hyphae were sometimes digested. The concentration of mycelium varied considerably. In most sections it was fairly abundant, but in others it could be located only with difficulty. There was no sharp increase in mycelial content in the outer central area though this was much more decayed than the adjacent dark zone, nor was there any concentration in the cells found to be decayed most rapidly. However, in the most decayed portions of the center, brown felts occurred, and little but mycelium was found when these were examined under the microscope.

Effects of the Fungus upon the Host Tissue

First effects of the fungus upon the wood were seen in the rays and xylem parenchyma at the outer limit of the dark zone. In this region there was a sharp increase in the number of dead cells, and the yellow granular deposit remaining in these became dark brown (Fig. 4), giving the outer area of the

infected wood its characteristic color. The masses in the parenchyma cells occasionally broke up into globules, and dark deposits formed in some of the vessels and fibers.

The precise nature of the dark masses in the cells could only be determined by chemical analysis. It was determined, however, that they were only partially extractable in boiling water or 85% alcohol, resisted bleaching with chorine water and choral hydrate, and failed to give any tannin reaction with ferric chloride. Moreover it was noted that the dark wood was less acid than normal wood. Dead cells in the rays of slightly discolored wood gave a yellow tinge to the neutral red solution used in vital staining indicating a pH of approximately 7.5, in contrast to the slightly acid reaction of adjacent living cells. Tests of aqueous extracts of the tissues were in close agreement with the determinations with neutral red, giving an average value of pH 6.3 for normal wood and pH 7.3 for the dark zone. In the light central area the alkaline reaction had disappeared and the pH of the water extract had dropped to 5.5.

The first action of the fungus upon the cell walls was noted a short distance back of the advancing edge of the brown zone where the larger and less lignified cells in the intervessel areas gave a reduced reaction with the Maule stain or phloroglucin (Fig. 3). Complete breakdown of cells was not seen in the dark zone, all of which appeared firm. Development of a spongy rot was coincident with the change to light color and acid reaction which marked the edge of the center zone. Just inside this zone the walls of the larger cells of the intervessel areas had become much thinner, and some had broken down completely. A similar thinning, followed by disintegration, took place more slowly in other cells of the wood. The vessels and surrounding fibers were gradually broken down and finally the rays and summer fibers. These were, however, very resistant to decay and were obtained in almost normal condition from tissue which could be crumbled in the fingers.

When sections of the center zone were mounted in the lignin stains the thin wall residues failed to stain (Fig. 10) but when treated with ruthenium red they became darkly colored (Fig. 9). Extraction with the pectic solvents previously listed gave no reduction in the intensity of stain, but served to differentiate the middle lamellae sharply from the secondary walls showing that the latter were digested first, the thin residues being entirely middle lamella. The disappearance of the entire secondary wall indicated that *P. glomeratus* had dissolved both lignin and cellulose, at the same time inducing changes in the middle lamellae which reduced their reaction to lignin stains and increased their affinity for ruthenium red.

It has not been the object of this investigation to carry the study of wall composition beyond the demonstration that different staining reactions occur which indicate variations in chemical composition. However, since the reaction to staining was found to be closely related to resistance to decay it may be concluded that the differences in composition are significant in development of the rot. Strongly lignified cells were found to be rather resistant to

decay, yet delignification, as determined by the phloroglucin reaction was the first stage of breakdown. Therefore, the pattern of decay cannot be explained simply by a preferential attack on either lignin or cellulose, and the nature of decay resistance of certain cells of the wood remains obscure.

Discussion

It has long been recognized that wound gum may form in response to invasion by parasitic fungi, and that it may have some protective effect (17). Gaumann (5) refers to "gummous demarcations" of the type described for *P. glomeratus* rot as "factors in antitoxic defense" protecting adjacent tissues from diffusing toxins and simultaneously inhibiting the growth of the causal organism. From the effect of *P. glomeratus* in advance of the mycelium it would seem that the wound gum is not an effective barrier to diffusion of toxic substances, but the reduced decay in the dark zone might be interpreted as showing that it has protective value.

Wound gum in undoubtedly a complex substance, and the manner in which it might affect decay can only be inferred from the present study. Development of a considerable amount of mycelium in the dark zone indicated that the gum is probably not toxic to the fungus, though a decrease in metabolic activity might affect the rate of spread and decay without reducing the final concentration of mycelium. From the evidence available the higher pH of the dark zone would appear to be the most probable cause of reduced decay, affecting the activity of the wall dissolving enzymes. A different reaction to pH of several of the enzymes produced by the fungus could explain the slow delignification in the dark, slightly alkaline zone, and the rapid breakdown of both lignin and cellulose following the change in reaction. Presumably the change in reaction occurs when sufficient acid has been produced to neutralize the alkaline component of the gum.

Data have been obtained (6) which indicate that no increase in pH occurs, and no wound gum is formed in autoclaved maple blocks overgrown by rot organisms, though the same fungi cause these effects in the living tree. The wound gum reaction is apparently dependent either upon living cells or upon relatively unstable compounds. Living cells of maple may, therefore, have a considerable influence on the development of heartrot even though they make up a small part of the wood. Since the number of living cells in heartwood undoubtedly varies from species to species (9), and perhaps from region to region, it would seem desirable to determine the 'vital content' of the wood in studies of decay of living trees.

Acknowledgments

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THE SPLASH-CUP DISPERSAL MECHANISM IN PLANTS[1]

By Harold J. Brodie[2]

Abstract

The late Prof. A. H. R. Buller regarded certain cup-shaped reproductive bodies found among cryptogams as "splash-cups" which make use of the energy of raindrops for the dispersal of spores, sperms, and gemmae. Buller's concepts are presented and supplemented by investigations carried out since his death. Raindrops cause the peridioles of the Nidulariaceae or bird's-nest fungi to be thrown about four feet by splash action. In the genus *Cyathus*, as a peridiole is jerked out of its cup, the funiculus is torn and this makes possible the expansion of a mass of adhesive hyphae (the hapteron) which clings to any object in the line of flight. The momentum of the peridiole causes a long cord to be pulled out of a sheath attached to the peridiole. The peridiole is checked in flight and the jerk causes the funicular cord to become wound around stems or entangled among plant hairs. Thus the peridiole becomes attached to vegetation and may be eaten subsequently by herbivorous animals. Several morphological features of fruit bodies of the Nidulariaceae are regarded as related to the utilization of the energy of raindrops in rain dispersal and the significance of the splash-cup principle is emphasized. Other splash-cups, the operation of which is described, are: the gemma cups of *Marchantia* and *Lunularia;* the antheridial disk of *Marchantia;* the leaf rosette of the male *Polytrichum* plant; the sclerotium cups of *Aleurodiscus Minnsiae;* the cupulate fruits of *Sagina decumbens* and *Oenothera rosea.*

Introduction

The ideas developed by the late Prof. A. H. R. Buller and reported as illustrated lectures delivered at various times and places during 1941–1943 concerning the so-called "splash-cup mechanism" by means of which spores of certain fungi, gemmae of certain liverworts, and sperms of certain mosses are dispersed in nature through the agency of raindrops were never published in detail and were recorded by him only in the form of a single short abstract (4).

Owing to close association with Professor Buller during the course of the investigations recorded in this paper, the writer has, for several years, been occupied in carrying Buller's plans to completion. Experiments have been repeated, observations verified, and new facts brought to light since Buller's death. It is hoped that publication of this material in detail may soon be possible. Meanwhile, a preliminary announcement of the principal observations and concepts seems warranted.

Until Buller (3) first drew attention in October, 1941, to the biological significance of the splash-cups of the cryptogams, there was little if any appreciation of the idea of splash dispersal. Many standard textbooks contained only brief and often vague reference to the dissemination of the gemmae of *Marchantia* or of the sperms of mosses by rain. Tulasne (10) had revealed the beautiful and complex structure of the fruit body of the Nidulariaceae but had not solved the problem of how the peridioles escape from it. Even when

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Contribution from the Department of Botany, Indiana University, Bloomington, Indiana, U.S.A.

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Martin (9), in 1927, reported what is apparently the first direct observation of the splash ejection of peridioles of the Nidulariaceae, there was no general recognition of the connection between the cup shape of certain reproductive structures and their function as what may be called splash organs.

In 1941, B. O. Dodge (5) gave an account of the discharge of the peridioles of the bird's-nest fungi based upon his own observations and upon those of Mr. John J. Shea. Dodge confirmed Tulasne's account (10) of the manner in which the funicular cord is extended in *Cyathus striatus* and pointed out that the cord serves to attach the peridiole to vegetation.

During the past decade, some recognition of the dissemination of the peridioles of the Nidulariaceae by rain has arisen (5, 8, 12) and in several recently published textbooks brief mention is made of splash dispersal in the Nidulariaceae, in *Marchantia*, and even among seed plants (7, 11). None of these accounts, however, emphasizes the complete significance of splash-cup dispersal nor gives much detail concerning the process.

The possibilities for future investigations revealed by Buller's research are numerous. Specialists working with mosses and liverworts may wish to follow some of the suggestions offered, and mycologists may be able to apply the concepts to groups of fungi other than those with which Buller was mainly concerned, namely the Nidulariaceae. With these points in mind, this preliminary paper has been prepared.

In some respects these researches do not include much material that is new. The morphology of many of the structures which Buller studied has long been known. As pointed out above, Tulasne (10) investigated and described in detail the fruit body of the Nidulariaceae or bird's-nest fungi as early as 1844, and Buller's investigations did not add greatly to the facts recorded by Tulasne. Likewise Buller's studies of the liverworts and mosses revealed few, if any, unknown details. However Buller did subject to very careful re-examination the various kinds of cup-shaped structures found among the cryptogams in the light of his concept of these structures as "splash-cups". He demonstrated experimentally that dissemination of the contents of the cups is accomplished in nature through the splashing action of raindrops, and he investigated the morphological peculiarities of splash-cups in various groups of plants in relation to their efficient operation in nature.

Although it is not possible at this time to present all the detail which is now available, the following is a brief account of the chief splash-cup types.

Some Splash-cups and Their Action

I. The Splash-cup in the Nidulariaceae or Bird's-nest Fungi

The funnel-shaped or vase-shaped fruit bodies of the Nidulariaceae (Figs. 3, 6) are splash-cups from which the peridioles containing basidiospores are ejected to a distance of three to four feet by the force of raindrops which land in the open mouths of the cups.

If the common species *Cyathus striatus* Pers. be taken as an example, the following points may be regarded as of importance in the operation of this cup in nature. The cup is about 6 to 8 mm. in diameter at the mouth. Large raindrops commonly have a diameter of about 4 mm. and these large drops are very effective in discharging the peridioles from a cup of comparable dimensions. The walls of the fruit body are elastic and resist being deformed by the battering of raindrops. Also, the fruit body is provided with a large solid mass of hyphae around its base which acts as an "emplacement" (Fig. 1) and prevents the splash-cup from being knocked over by rain.

When large raindrops, having a terminal velocity of about six meters per sec. fall into the fruit body, the displacement of the water in the cup creates a strong thrust upward along the inclined sides and the peridioles are forcibly ejected (Fig. 2, C). The splash-cup has sides which form an angle of 60°–70° with the horizontal. This angle is probably related to the splash action, for it can be shown that the maximum lateral ejection of water drops from model cups is observed when such cups are constructed with the sides inclined at an angle of the same order.

As is well known, the basidiospores in the fruit body of a member of the Nidulariaceae are contained in lenticular capsules called peridioles. In *Cyathus striatus* each peridiole is attached to the inner wall of the fruit body by a stalk called the *funiculus* which has a complex structure consisting of a *sheath*, a *middle piece*, and a *purse* (Fig. 1). When a peridiole is being splashed from a fruit body, the force jerks the funiculus, and the purse is torn open at its lower end where it is attached to the middle piece. The rupture of the purse causes the almost instantaneous expansion of a special mass of hyphae that had been coiled up under tension within the lower part of the purse. The hyphae are highly adhesive and form an attachment organ or *hapteron* (Fig. 1).

When the peridiole in flight from the fruit body strikes an object such as a leaf, petiole, or stem, the hapteron, consisting of a mass of loosely interwoven hyphae, becomes entangled in epidermal hairs and other protuberances (Fig. 2, D and Fig. 4). The peridiole may then continue in flight and, as it does so, there is pulled out from the upper part of the purse the long funicular cord (Fig. 1).* This cord is of the nature of a cable, being composed of a hundred or more hyphae spirally twisted about one another. By virtue of its structure, the cord has considerable strength and elasticity. It may occasionally be extended as much as three inches. When the hapteron end of the cord becomes caught, the peridiole is checked in its flight by the funicular cord and is jerked backward (Fig. 2, E). As a result, the peridiole tends to swing around its attachment point and thus to become securely fastened to whatever object it may have struck (Fig. 2, F). Even when the peridiole glides along an object such as a leaf, it easily becomes attached to that object because of the adhesive nature of the hapteron (Fig. 4).

** It is possible that the funicular cord is pulled out as the peridiole leaves the fruit body. Although there is considerable evidence to indicate that this does not occur and that the cord is extended only after the hapteron becomes attached to some object in the line of flight, the question could be answered only by stroboscopic photographs of the peridiole in flight.*

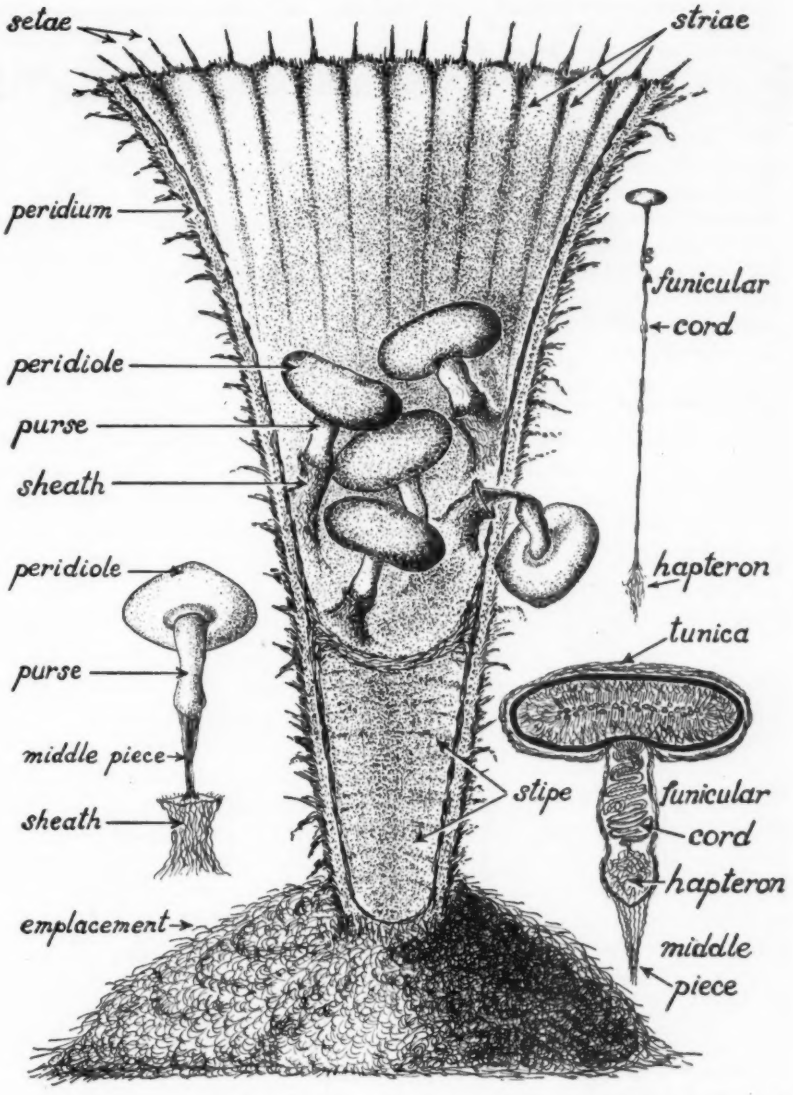
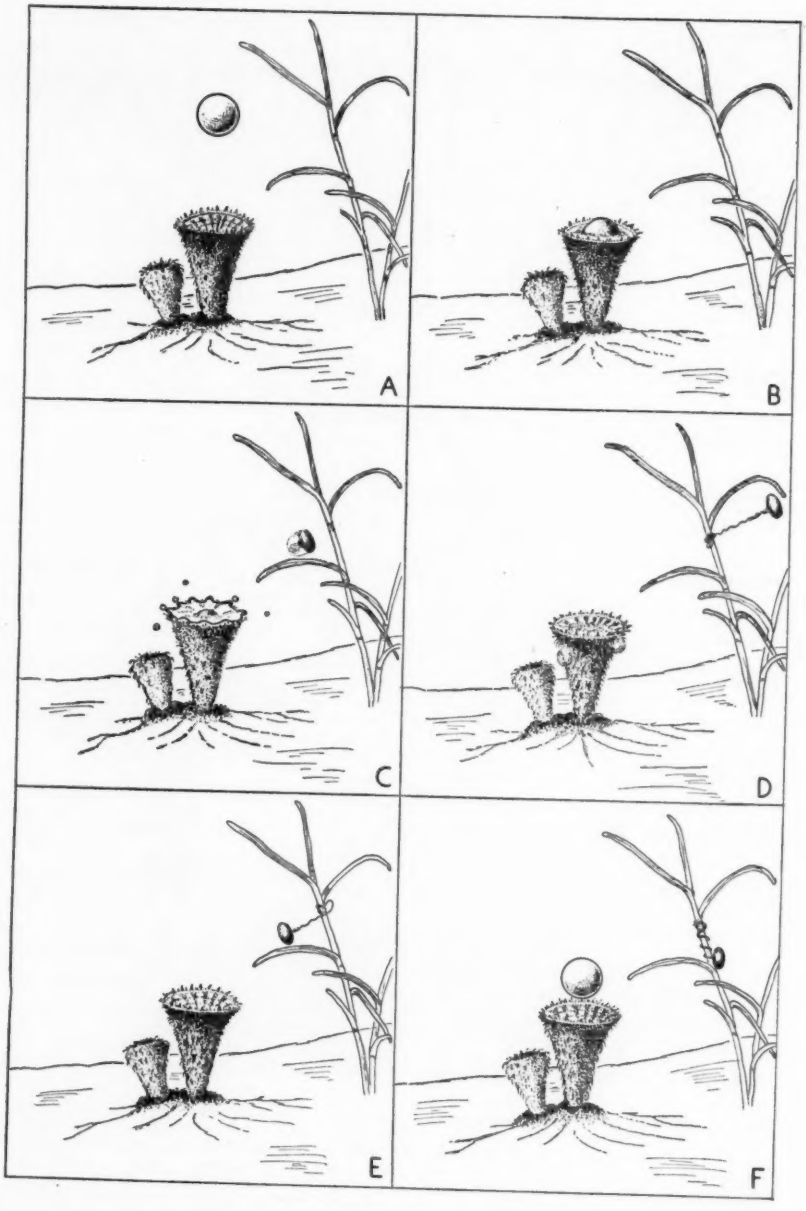


FIG. 1. Section of fruit body of *Cyathus striatus* (semidiagrammatic), showing names of various structures referred to in the article. Spores are borne inside the lenticular peridioles, each of which is attached to the inner wall of the fruit body by a funiculus consisting of sheath, middle piece and purse. When a raindrop ejects a peridiole, the purse is torn open at its lower end freeing the long funicular cord. Central figure ×10 approx. Section at right ×20 approx.


A
B
C
D
E
F

Having become attached to a leaf or stem, the peridiole remains there. When the adhesive hapteron dries, it cannot easily be washed off. The subsequent fate of the peridiole was partly conjectured by Buller by analogy with what he knew of *Sphaerobolus* and *Pilobolus*, which shoot adhesive spore masses on to vegetation where they remain until the vegetation is eaten by horses, cows, and other herbivorous animals. It cannot be said with certainty that the peridioles of all members of the Nidulariaceae eventually pass through the alimentary tract of some animal, but we have considerable evidence to suggest that they do. Investigations concerning *Cyathus stercoreus* (Schw.) De Toni have shown (Brodie (1)) that the basidiospores of this coprophilous species can be germinated only with difficulty unless they are heated, which might indicate that they are heated in nature in the alimentary tract of an animal or subsequently in the decomposing faeces. Furthermore, it has been observed that the enzyme lipase acts upon the hard cortex of a peridiole to soften it and allow the basidiospores to escape from inside, and this may be what happens to the hard peridiole wall in nature. Finally, there is great variation in nature in the form and color of the fruit body of *C. stercoreus* (Brodie (2)) which would not be expected unless the basidiospores were eventually freed from one another so that haploid mycelia having different genetic constitutions could be widely distributed in nature.

Peridioles of the Nidulariaceae variously attached to vegetation by their funicular cords and haptera can easily be found by anyone who troubles to examine foliage and stems within two to four feet of a fruit body after a heavy rainstorm (Fig. 4).

Professor Buller experimented with several species of the Nidulariaceae and the writer has since repeated his work and extended it. By allowing drops of water to fall far enough so that they reach their maximum terminal velocity (about 9 ft.) and causing the drops to fall into the open mouths of the fungus cups, Buller demonstrated that the peridioles are very effectively splashed out of the cup to distances up to four feet. In recent investigations, the writer has extended the distance to seven feet using *C. stercoreus* but has never seen peridioles shot farther.

Early investigators, finding the funicular cord neatly coiled within the purse surmised that the cord might constitute a spring mechanism by means of which the peridiole might be shot out. We can now conclude, however, that the sole force acting to eject the peridioles is that resulting from the kinetic energy of falling raindrops.

FIG. 2. Diagram of the splashing of a peridiole from a fruit body of *Cyathus striatus:* A and B, raindrop lands in cup; C, splash action ejects peridiole which flies out with adhesive hapteron extended; D, hapteron sticks to plant stem as peridiole is carried forward by its own momentum and funicular cord is extended by pull; E, peridiole is jerked back when it reaches limit of extension of cord; F, jerk causes peridiole to swing around point of attachment and cord is wrapped around plant stem as another raindrop lands in splash-cup. ×3/2.

Not all members of the Nidulariaceae have as complex a fruit body and funiculus as has *C. striatus*. In *Crucibulum vulgare* Tul. the peridiole is attached to the fruit body by a funiculus, but the latter is simpler than that of *Cyathus striatus* just described. In the genus *Nidula* there is no funiculus and the peridioles are covered with an adhesive substance which sticks them to vegetation. In *Nidularia* the peridioles are likewise devoid of funiculi and provided with an adhesive coating.

In the shape of the fruit bodies there may be noted a graded series of forms regarding splash-cup development. *Nidularia* has globose fruit bodies that break open irregularly at the top and are not efficient splash organs. *Crucibulum* fruit bodies are massive and the angle of the sides of the cups varies considerably, both features seeming to detract somewhat from the efficiency of this type as a splash organ. In the common species of *Cyathus* the highly developed vase-shaped fruit bodies are the most efficient splash organs thus far investigated.

II. The Gemma Cups of *Marchantia* and *Lunularia*

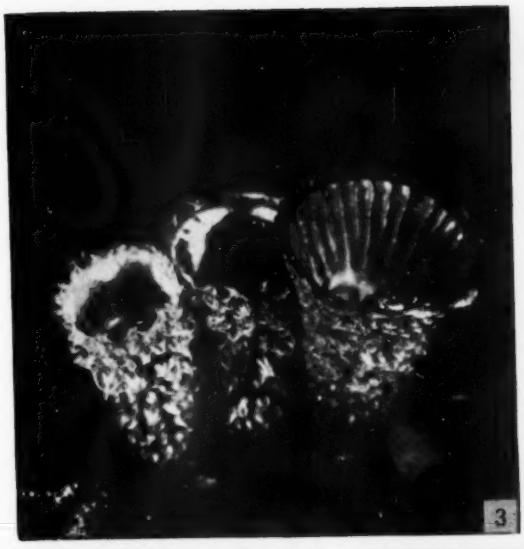
The gemmae of *Marchantia polymorpha* L. which serve for asexual reproduction are developed in small cupules broadly attached to the thallus (Fig. 5). It is generally accepted that gemmae are washed out of the cupules by rain and it is sometimes stated that they may be splashed out, but it is not generally realized that this liverwort cupule is a splash-cup mechanism.

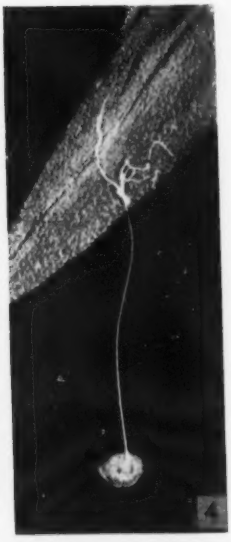
Following a suggestion found in Professor Buller's notes, the writer has demonstrated that the gemmae may be shot as much as two feet from the cupule when small raindrops fall into it. To mention only two points in which the cupule is comparable with the splash-cup of a bird's-nest fungus, the cupule has sides which form an angle of 60°–70° with a horizontal and the gemmae are lenticular. The general similarity of the splash-cups of *Marchantia* and *Cyathus* when seen from above is shown by a comparison of Fig. 5 with Fig. 6.

In *Lunularia cruciata* (L.) Dum. the gemma cup is crescent-shaped or is a half cup, the opening being directed outward, away from the thallus. Professor Buller himself experimented with *Lunularia* and showed that the gemmae may be splashed radially away from the thallus by raindrops to a distance of two feet. In view of the fact that the cupules of *Marchantia* and *Lunularia* are but 2 to 3 mm. in diameter, the distance to which their gemmae are dispersed by splashing is remarkable.

Fig. 3. *Cyathus striatus* splash-cups, side view. ×5. Fig. 4. Peridiole of *C. striatus* attached to leaf by means of funicular cord, the hapteron end of which is seen entangled in epidermal hairs of leaf. The specimen was photographed exactly as found in nature after a heavy rainstorm, the peridiole at 3 ft. 2 in. from the nearest fruit body. The funicular cord is not taut because peridiole lies on cardboard used as background for photograph. ×4. Fig. 5. Splash-cups of *Marchantia polymorpha*. These are the gemma cups which contain lenticular gemmae seen in lower figure. This photograph, courtesy of Prof. E. B. Mains. ×17. Fig. 6. The splash-cup of *Cyathus striatus*, as seen from above, containing peridioles. Note general similarity between this and lower cup of Fig. 5. ×7.

PLATE I





III. The Antheridial Disk of *Marchantia*

In *Marchantia polymorpha* sperms are developed in antheridia imbedded within a saucerlike platform which surmounts a stalk 1 to 1.5 in. high, and archegonia are developed on the lower sides of the fleshy rays of the archegoniophore. As antheridia rupture when wetted, the sperms ooze out on to the disk of the antheridiophore. Buller allowed small drops of water to fall three feet on to antheridial disks bearing freshly discharged sperms and observed that drops of live sperms were splashed radially away from the disk to a distance of from 18 in. to two feet. He therefore considered that the disk of the liverwort *Marchantia* is a special kind of splash-cup which he referred to as a "splash-platform". Although the platform is saucerlike rather than cuplike, its edges are flared upwards sharply, and it seems to operate essentially as do the more cup-shaped or vase-shaped splash-cups of *Cyathus* and the *Marchantia* gemma cupule.

While it cannot be denied that many sperms must be washed from the antheridial disks in nature, rather than splashed, it would seem reasonable to assume that those that are splashed would have a better chance of reaching archegonia than those that are washed on to the ground whence they would have to swim up the archegoniophores to reach archegonia.

IV. Splash-cups in the Mosses

The top of a male plant of *Polytrichum* consists of a rosette of specially modified stiff leaves which form a cup varying in diameter from 2 to 3 mm. in some species to about 6 mm. in others. Within the cup, antheridia are developed. When the antheridia burst, sperms are liberated and accumulate in masses in the moss cup. Again raindrops serve to splash sperms from the cups. Professor Buller made no observations on mosses, but the author recently experimented with male plants of *Polytrichum ohioense* R. and C. and found that, although the splash-cup in this species is small (2 to 3 mm.), sperms are easily splashed as much as two feet by small water drops falling only three feet into the cup. It is probable that sperms may be splashed even more effectively from the larger cups of *Polytrichum commune* L. Also, the cuplike structures surmounting male plants or male branches in other mosses may be capable of acting as splash-cups, although these have not been studied.

In the moss *Tetraphis* (or *Georgia*) the shoots are surmounted by rosettes of leaves and within the cup so formed there lie gemmae for asexual reproduction. Buller suggested that, here again, we have to deal with a splash-cup. Examination of the gemma-bearing cups of *Tetraphis* does not convince one that they are actually efficient in splashing, partly because of their small size and partly because of the rather delicate character of the plants which are easily battered about by raindrops.

V. The Cups of the Lichens

In certain lichens such as *Cladonia* spp. there are produced symmetrical cups that bear a striking resemblance to those of *Cyathus*. These cups (or podetia) contain reproductive units called soredia which consist of some cells of the alga component and some hyphae of the fungus component.

Professor Buller did not give any special attention to lichen cups. However, it is easy to demonstrate that the soredia can be splashed as much as three feet from the cups, especially if one experiments with the most funnel-shaped specimens. Since soredia when dry can also be blown from the podetia by wind, it may be that cups in the lichens are not to be regarded as splash mechanisms. A profitable field of investigation would seem to be suggested by the podetial cups in this group of plants.

VI. Other Splash-cups Among the Fungi

It is obvious that not all cup-shaped or vase-shaped reproductive bodies in the fungi (or other lower plants) are splash mechanisms. For example, ascospores are puffed from the fruit bodies in discomycetous fungi, and it is doubtful whether spores could be splashed effectively from such cups which are frequently too large for this type of dissemination.

However, in certain other fungi, structures have been found which do suggest the splash mechanism. Prof. H. S. Jackson has recently described (8) a species of *Aleurodiscus* (one of the Thelephoraceae) in which a cupulate body is developed, usually on the upper sides of twigs. Within each cup there lies a single spherical mass of mycelium which is a sclerotium that serves for asexual reproduction. Professor Jackson suggested that the sclerotium cups function as splash-cups and kindly sent to the writer a fresh collection of *Aleurodiscus Minnsiae* Jackson in September 1950. Although the sclerotial cups in this fungus are minute, they do act as splash organs for it was found possible to splash sclerotia as much as three feet from their cups by means of small water drops.

VII. Splash Dispersal Among Flowering Plants

From the above account it will be apparent that the idea of splash dispersal invites a re-examination of many kinds of cupulate fruits of flowering plants. Many of the standard treatises on morphology refer to the possible dissemination of seeds by rain, but there has probably been no general recognition of special structures as splash-cups. Two examples only of splash mechanism may be noted.

1. *Sagina decumbens* (Ell.) T. and G. Gibbs (7) has recently drawn attention to the fact that the capsules of this species of pearlwort are cupulate and open wide only when wetted. He notes that small drops of water "falling from a height of only 6 inches, splashed the seeds to a distance of up to 2·5 inches from the capsule." The present writer also examined the splash-cups

of this plant, specimens of which were provided by Mr. Charles Deam, and can confirm Professor Gibbs' report. In one trial, six seeds were found to have been splashed 18 in. from a capsule struck by a large water drop which fell through six feet.

2. *Oenothera* (*Hartmannia*) *rosea* Aiton. A colleague, Dr. Charles W. Hagen, Jr., suggested that the capsules of this plant might constitute a splash mechanism because they are closed when dry and open only when wetted. Using specimens provided by Dr. Hagen it has been observed not only that the capsules are strongly hygroscopic, but also that seeds can be splashed as much as three feet from the open capsule. The central stalk which projects upward from between the open valves does not seem to interfere with efficient splashing.

VIII. Mechanics of Splash Dispersal

As yet, a detailed analysis of the mechanics of splash-cups has not been attempted. A number of facts appear, however, to be related to the utilization of the kinetic energy of raindrops in splash dispersal. The most efficient naturally occurring splash-cups have these features in common: (1) the cups are funnel-shaped with sides sloping about 60°–70°; (2) the cups are mostly 5–7 mm. in diameter at the top; (3) the cups are broadly attached at the base; (4) the bodies dispersed from the cups are lenticular in shape.

A raindrop 4 mm. in diameter plunging into the rain-filled fruit body of *Cyathus striatus* may act piston-wise to force water out along the inclined sides of the cup. This thrust would be received broadside by the lenticular peridiole which would then be thrown from the cup by the force.

High-speed photographs of splashes which are formed when a drop of milk falls on to the surface of milk in an open vessel published by Edgerton and Killian in 1939 (6) show that the liquid which is thrown up by the splash assumes the shape of a crater or funnel. The resemblance of such splash craters to the shape of a fruit body of *Cyathus striatus* or of the gemma cup of *Marchantia* is impressive and indicates that the shape of splash-cups in nature bears a close relationship to the splash pattern in general.

General Remarks

Although the various kinds of splash-cups described herein have long been well known to botanists, the concept that such cups represent efficient organs for the dissemination by rain of spores, sperms, gemmae, etc., does not seem to have been fully appreciated nor sufficiently emphasized in the past.

As indicated above, the first observation of the actual splash ejection of peridioles of the Nidulariaceae appears to have been that of G. W. Martin in 1927 (9) but there are several suggestions of the possibility in the older literature. For recognition of the splash-cup principle we are indebted to the late Professor Buller, for it was he who first appreciated its full significance and began experimental study of the kind indicated in the present paper and which it is hoped to be able soon to present in detail.

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TOBACCO ETCH VIRUS IN ONTARIO[1]

By R. H. Stover[2]

Abstract

Tobacco etch is one of the most widespread and injurious viruses on burley tobacco in Ontario. Three strains of this virus were obtained from tobacco collections. Their symptoms and behavior on tobacco and other hosts are described. Tobacco varieties were divided into two groups on the basis of their reaction to the etch virus. The group comprising all burley varieties tested showed severe symptoms while the other, comprising flue cured, dark, and cigar varieties, developed mild symptoms. The F_1 from severe-symptom varieties × mild-symptom varieties exhibit mild symptoms while the F_2 has segregated into severe- and mild-symptom types. The widespread occurrence of etch is attributed to the prevalence on tobacco of the vector *Myzus persicae*. A mixture of potato virus X (ringspot strain) and etch causes a more severe disease on burley tobacco than either virus by itself.

Introduction

During the past three years, certain virus diseases have been epidemic in the burley tobacco belt (Essex and Kent counties) of Ontario. In some areas these viruses have caused considerable injury and impaired the quality of the leaf. Circumstantial evidence indicated that the aphid *Myzus persicae*, which first appeared on tobacco in Ontario in 1947, was a vector of the viruses in question. This evidence, in addition to the kind of symptoms encountered, strongly suggested the cucumber mosaic or tobacco etch viruses as the major causes of the epidemic. Preliminary tests on differential hosts, however, soon established tobacco etch as the most common virus present in the numerous collections that were made, followed by potato virus Y (vein-banding), two strains of potato virus X, cucumber mosaic, tobacco streak, and tobacco ring spot. Tobacco mosaic (several strains) was common in all fields examined at the topping stage. The streak and tobacco ring spot viruses were common but relatively few plants were infected in any given field.

Three different strains of etch were obtained from the tobacco collections of which two appear to be different from the strains previously described. Since the present paper constitutes the first report of the etch virus on tobacco in Canada, the symptoms and behavior of the various strains on commercial varieties of tobacco in Ontario are described.

Review of Literature

The disease caused by the etch virus on tobacco was first described by Valleau and Johnson (18) and Johnson (13) in 1928 and 1930. They (13) found four strains of the virus in Kentucky, Georgia, and Ohio, and suggested

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(19) that potatoes may be a source of inoculum. In 1937, Chester (6) reported that the etch viruses formed a distinct serological group. Kassanis (14), in 1939, described characteristic intranuclear inclusions in plants infected with etch. The formation of these inclusions, and those occurring in the cytoplasm, were studied by Sheffield (17) in 1941 and described further by Bawden and Kassanis (2).

The properties of this virus group were described by Bawden and Kassanis (2). They found the dilution end point to be approximately 1/5000 for severe etch and 1/1000 for the mild etch strains. The thermal inactivation point was 58° C. for 10 min., and between 52° and 58° C. infectivity was greatly reduced. After five days' storage at room temperature, the infectivity of severe etch virus had fallen to less than 1/100 of the original, but complete inactivation was not obtained until after 13 days. McKinney (16), using a special technique of desiccation, found that etch was infective in dry tissue after 301 days. Bawden and Kassanis (2) described the effect of trypsin on the virus and methods for partial purification. These workers (3) described also the suppression of multiplication of potato virus Y and *Hyoscyamus* virus 3 by severe etch and discussed the possible explanation of these phenomena and the similarities of these three viruses. Bawden (1, p. 231) states that he and Nixon, using the shadow-casting technique, have determined the rod-shaped particle width of etch to be approximately 10 mμ and of variable length.

The Rothamsted workers have investigated the vector relationships of etch. Kassanis (15) studied the transmission of these viruses by several species of aphids, and Bawden (1) lists the following as vectors: *Aphis fabae* Scop., *Aphis rhamni* Fonsc., *Aulacorthum circumflexum* (Buckt.), *Macrosiphum euphorbiae* (Thomos), *Myzus ascolonicus* Doncaster, *Myzus persicae* (Sulz.). The vector relationship was found to be typical of the nonpersistent virus group (15).

The host range of severe etch was studied extensively by Holmes (12) with the aid of a local lesion host (*Physalis peruviana*) (9). Holmes (10) describes the use of this host for quantitative studies, and factors affecting the development of lesions. Holmes (11, 12) was able to secure infection on more than 69 species of plants in 11 families and found that all species infected by etch were also hosts of tobacco mosaic.

Fulton (8) has described the behavior of etch and other viruses in plant roots, and their movement through the plant. He (8) found the thermal inactivation point of the etch virus to be 60° C. in leaf extract and 57° C. for 10 min. in the roots. Bennett (4) found that, of several dodder species tested, only *Cuscuta californica* transmitted etch to Turkish tobacco. He (4) described the behavior of etch and several other viruses in various species of dodder. In a subsequent publication, Bennett (5) states that, when tomato plants which had recovered from the effects of dodder latent mosaic were infected with etch, by way of *Cuscuta californica*, the symptoms of the former virus reappeared. The plants then remained dwarfed and continued to show

signs of dodder latent mosaic. Doolittle (7) reports that severe etch causes a serious disease of sweet peppers in New Jersey and Maryland. Holmes (11) found the perennial weed *Physalis subglabrata* to be naturally infected in New Jersey.

Materials and Methods

Eight species of *Nicotiana* and 18 varieties of *N. tabacum* were used as hosts for studying the viruses, in addition to tomato (Bonny Best), pepper (Penn Wonder), *Datura stramonium*, eggplant (Black Beauty), cucumber (Straight 8), sweet pea, zinnia (Crimson Monarch), bean (Robust), *Vicia faba*, potato (President, U.S.D.A. 41956, Irish Cobler), *Gomphrena globosa*, and petunia (Rosy Morn).

Tobacco plants were grown in 4-in. pots and inoculated when the leaf rosette was 4 in. or more in diameter. Applications of fertilizer were made periodically to ensure continuous growth. Auxillary lighting in the form of fluorescent daylight lamps was used from November to March to approximate a 15-hr. day. Inoculations were made by crushing infected leaf tissue in a small amount of tap water and smearing the leaf with a cotton swab stick or the forefinger. In some instances carborundum was used.

Cross protection tests were carried out with a strain of tobacco mosaic that induces local lesions in varieties of burley tobacco carrying the *N'* gene, and with Fulton's cucumber strain C from zinnia. Where possible, cross protection was tested with the different strains of etch (Table III).

Symptoms

It was apparent, after numerous series of inoculations, that at least three distinct strains of etch were present in the Ontario virus collections. However, only one of the strains caused characteristic etching consistently on burley varieties and Samsun. This strain caused symptoms on burley tobacco almost identical with a specimen of etch obtained from Dr. Valleau in Kentucky and designated by him as "etch". The Ontario etch strain will, therefore, be designated as Strain K in the present studies because of its similarity to the Kentucky strain. It is believed that Strain K is similar or identical with the "severe etch" strain described in the literature. It caused characteristic etching on burley varieties, and the production of intranuclear inclusions similar to those described by Kassanis (14) and Sheffield (17). The other two Ontario strains studied did not usually produce any etching symptoms in the greenhouse, and numerous tests were required to establish their relationship to Strain K. These strains are designated as N and V. In the field, Strain N caused a strong necrotic response on burley tobacco (Fig. 6) and Strain V, pronounced vein-clearing. The symptoms, differentiation, and relationships of these three strains are described in Tables I, II, III, and the text.

TABLE I

SUMMARY OF THE RESPONSE OF DIFFERENTIAL HOSTS TO THE ONTARIO STRAINS OF ETCH VIRUS

Strain	*N. tabacum*		*Datura stramonium*	Pepper var. Penn. Wonder
	Severe-sympton varieties*	Mild-symptom varieties*		
K ("severe etch")	Vein-clearing, chlorotic mottle, pronounced etching, stunting. Necrotic lesions on roots. Severe chlorosis of rapidly growing leaves	Faint vein-clearing and mild mottle; etching and other symptoms mild	Vein-clearing, chlorotic mottle, distortion, and stunting (Fig. 10)	Symptoms variable. Dark green mottle or chlorotic cast, chlorotic rings, sunken ring spots on fruit. Some plants only mild or masked mottle. May be stunting and leaf distortion
N	Mild mottle in upper leaves, necrotic spots on lower and middle leaves after 4 weeks. Necrosis of upper leaves in field (Fig. 6)	Very mild indistinct mottle, chlorosis, and wilt of old leaves on mature plants	Severe leaf distortion, rugosity, and stunting (Fig. 11)	Symptoms masked or no infection, old plants cut back sometimes produce mottled suckers with mild distortion
V	Mild often indistinct mottle in form of circular or diffuse chlorotic spots, faint vein-banding. Pronounced vein-clearing and necrotic line patterns in field (Fig. 14)	Very mild, indistinct pale green mottle; faint vein-banding	Mild chlorotic spot mottle, becomes masked, often no visible symptoms	No symptoms observed

* *See Table II.*

TABLE II

RESPONSE OF *N. tabacum* VARIETIES TO STRAIN K ("SEVERE ETCH") IN THE GREENHOUSE

Severe-symptom varieties	Mild-symptom varieties
Burley Haronova Harrow Velvet Judy's Pride Halley's Special Green Briar White Burley Kelley	*Flue cured* White Mammoth Yellow Mammoth
Turkish Samsun Samsun (Turkish)	*Dark* Little Crittenden Greenwood
	Cigar Connecticut Havana 38*
	Other types Ambalema* T.I. 448A*
	Halley's Special × Delcrest F_1 Harrow Velvet × Delcrest F_1

* *These varieties showed more pronounced symptoms than the other mild symptom varieties.*

TABLE III

CERTAIN PROPERTIES OF THE ONTARIO STRAINS OF ETCH VIRUS

Strain	Transmission by *Myzus persicae*	Thermal inactivation*	Cross protection tests**	Intracellular inclusions
K ("severe etch")	+	59° C.	—	Characteristic (17), crystalline plates abundant in nuclei and cells
N	+	56°-59° C.	N protects against K only if fully infected leaves are inoculated	No intranuclear inclusions but large, granular, amorphous cytoplasmic inclusions. About same size as nuclei or larger and often adjacent to them
V	+	55°-59° C.	V protects against K and N	No intranuclear or cytoplasmic inclusions observed in greenhouse material

** One-inch squares of infected leaves were immersed in a water bath for 10 min.*

*** Carried out on varieties Green Briar and Harrow Velvet inoculated two weeks previously with the "protector" strain.*

SYMPTOMS CAUSED BY STRAIN K ON *N. tabacum* IN THE GREENHOUSE

(*a*). *On Varieties Harrow Velvet, Judy's Pride, Halley's Special, Green Briar, White Burley, and Kelley*

From 7 to 12 days after inoculation, vein-clearing is present in the young, expanding leaves. This is followed by a chlorotic or dark green mottle, and etching that is often confined at first to the basal half of some leaves (Figs. 1, 9). Severely infected leaves have a pronounced chlorosis and yellow mottle. Considerable variation in symptoms may be observed on plants of the same variety because of the different physiological states that exist in the host at different times of inoculation. Considerable fluctuation in environmental conditions during the winter months in the greenhouse contributes to the range of symptom expression. Mottled leaves may or may not show etching. Chlorosis may be severe or mild but is usually more pronounced than that occurring on flue-cured varieties. The etching consists of white or gray irregular lines, circles, flecks, and variously shaped curves and arcs (Fig. 2). On young, systemically infected seedlings that are growing rapidly, there is a general chlorosis of the plant accompanied by stunting. After six weeks Harrow Velvet plants infected with etch were only one-half as large as the checks. The etching symptom may recede, or fail to develop, only to become intensified at a later stage of growth. Necrotic spotting often appears on mature leaves. An examination of the roots of plants three or four weeks after infection indicates the presence of numerous small areas of necrosis on some of the roots. The cells in these areas are brown or black and the contents are granular. The characteristic, intracellular inclusions described by Sheffield (17) can be easily found in the roots and tegumentary cells of infected leaves.

(*b*). *On Varieties Little Crittenden, White Mammoth, Yellow Mammoth, Delcrest, Greenwood, Connecticut Havana 38, T.I. 448A, Ambalema, and Oxford 400*

Symptoms on these varieties usually appear several days later than those on burley tobacco. The first symptoms consist of mild vein-clearing and a diffuse, faintly chlorotic and dark green mottle on the young leaves. This is followed by mild, fine etching on the inoculated leaves, which may be accompanied by necrotic flecking. Etching often does not appear on subsequently formed leaves and, if present, consists of very fine gray lines on a dark green and chlorotic mottled background. The etching is not associated with the severe chlorosis that usually occurs on burley varieties infected with this virus. In fact, chlorosis on these varieties is very mild in contrast to that occurring on burley tobacco. There is little distortion and stunting and all symptom expressions are much milder than those on the burley types. However, there are varietal differences within the group with respect to the intensity of symptoms. T.I. 448A, Oxford 400, and Connecticut Havana 38 show more pronounced symptoms than the other flue cured and dark tobacco varieties tested.

(*c*). *On Variety Samsun*

The first symptoms of mild vein-clearing are similar to those on burley tobacco. The chlorotic mottle that follows is usually milder and more spotty. Chlorosis is less severe than on the burley varieties. Etching is very pronounced in the early stages of plant growth (Fig. 2) and may disappear in later stages. In some instances, symptoms on this variety are variable and only a mild, diffuse, chlorotic mottle appears. Chlorotic, circular spots up to $\frac{1}{8}$ in. in diameter are sometimes pronounced on the upper leaves. There may be mild leaf distortion and stunting.

Symptoms Caused by Strain K on *N. tabacum* in the Field

(*a*). *On Variety Harrow Velvet*

Symptoms on naturally infected plants in the field were usually first noticeable as the plants approached the topping stage and became intensified after topping. Vein-clearing, with or without necrosis, was prominent on the lower leaves. The most common symptoms were necrotic spotting of the upper leaves (Fig. 5), and a diffuse chlorosis of the leaves, which finally becomes necrotic. The necrosis often formed oak-leaf patterns. Severely affected

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Fig. 1. A leaf of burley tobacco showing mottling and etching caused by Strain K.

Fig. 2. A lower leaf from a young plant of the Samsun variety with etching caused by Strain K.

Fig. 3. A lower leaf of the Samsun variety showing the necrotic spotting that develops about four weeks after inoculation with Strain N. This spotting occurs also on burley varieties to a greater or less degree.

Fig. 4. An upper leaf from a burley variety showing the characteristic chlorotic spot mottle caused by Strain N. A similar mottle develops on the Samsun variety and the necrosis shown in Fig. 3 usually does not affect the upper leaves.

PLATE I

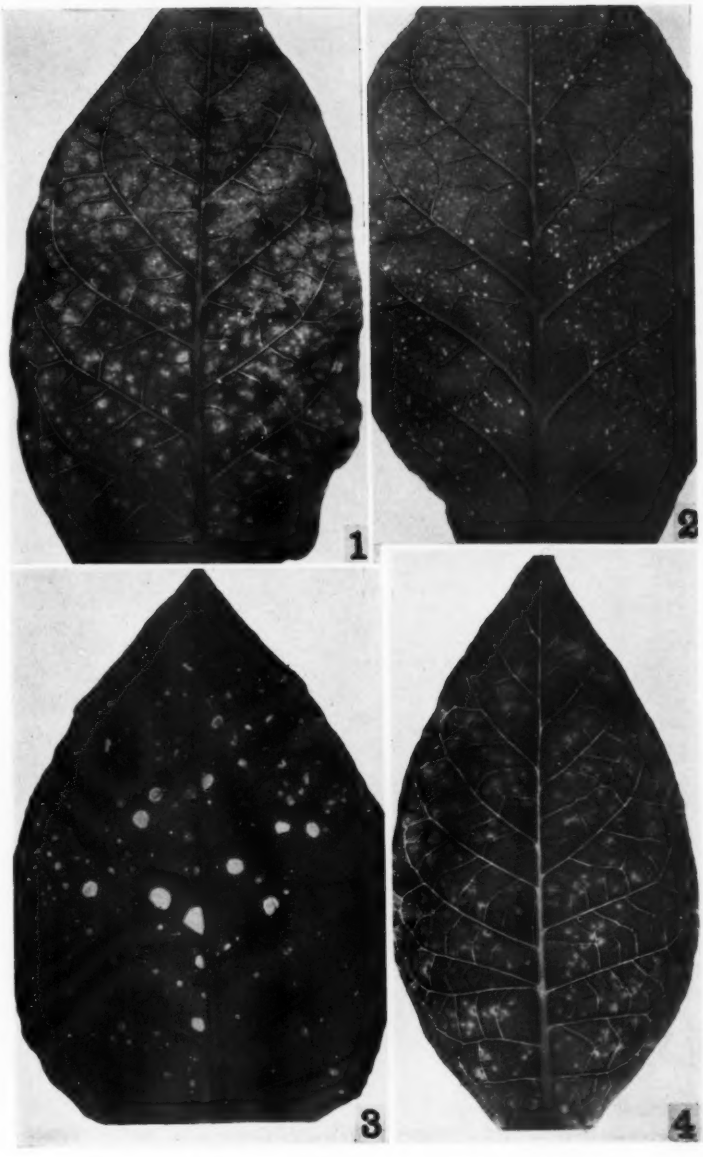
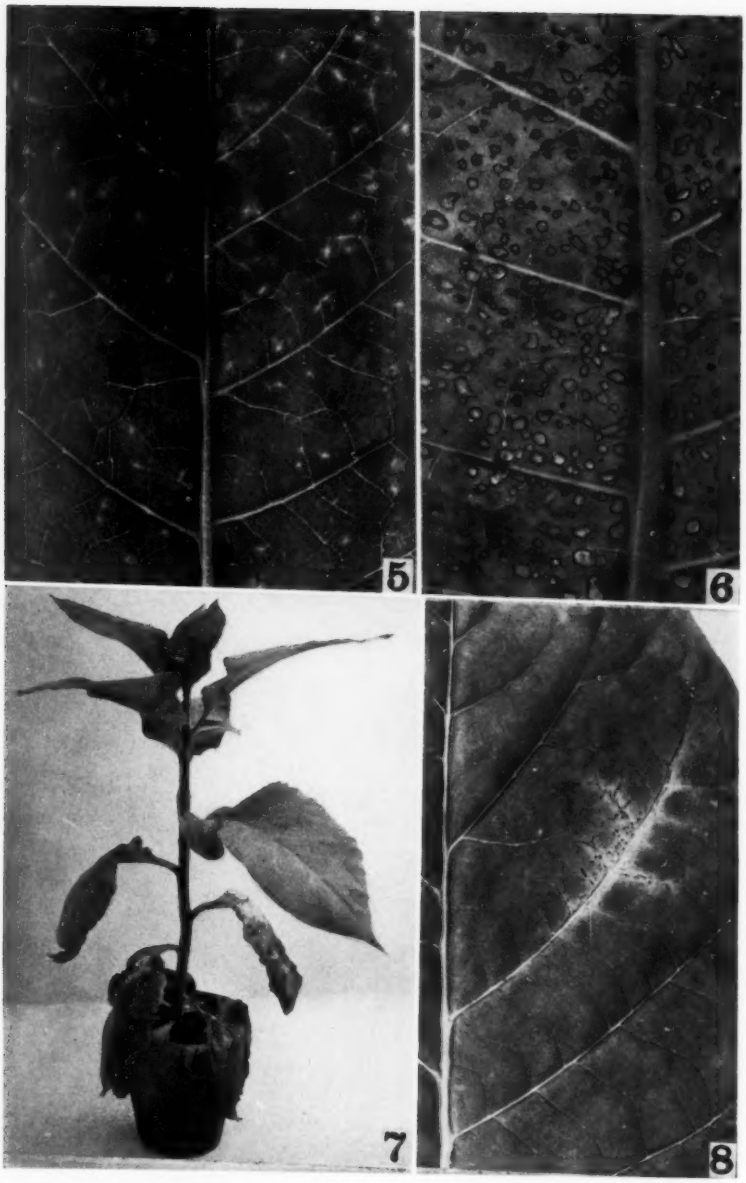


PLATE II



fields contained "fired", chlorotic, and tattered upper leaves at the harvesting stage. Cigarette type burley (Harrow Velvet, Haronova) was more severely injured than the export or wrapper type (Green Briar, Kelley).

(*b*). *On Variety Little Crittenden*

No pronounced primary symptoms were visible on naturally infected field plants. After topping, numerous small necrotic spots (Fig. 13) developed along the veins of the large mature leaves. A distinct mottle was evident only on the sucker growth. There was no stunting or leaf distortion, and in the absence of mottling, symptoms were identical with those of certain physiological leaf spots. An average of two or three leaves near the top of the plant showed severe symptoms.

SYMPTOMS CAUSED BY STRAIN K ON HOSTS OTHER THAN *N. tabacum*

(*a*). *On Datura stramonium*

Vein-clearing followed by a mild, chlorotic mottle are the first symptoms. There is some narrowing and rolling of the leaves with mild distortion (Fig. 10). Dark green vein-banding may occur on old plants.

(*b*). *On Pepper var. Penn. Wonder*

Symptoms are most variable on this host. A dark green mottle usually develops first and this may be very faint. As the plant matures and bears fruit, the top leaves may have a chlorotic cast with an indistinct mottle. The mottle may consist of small chlorotic rings and spots up to $\frac{1}{4}$ in. in diameter. Necrotic spots about $\frac{1}{8}$ in. in size may develop, with traces of necrosis in some veins. Leaf abscission may occur. The fruit may be misshapen and stunted with an irregular surface. Fruit symptoms may consist of small necrotic flecks, and indistinct, slightly depressed ring patterns may be visible. After the fruit reddens it may shrivel on one side. In some instances the only symptoms are a mild mottle and leaf distortion. On many plants the symptoms were masked and either developed after the plant was cut back, or not at all. In a few plants, a stunted, bushy growth developed with numerous suckers and distorted leaves.

As the symptoms on the other hosts were not studied in detail and have been reported by Holmes (12), they are not included in this paper. All species of *Nicotiana* tested became infected, as well as tomato, petunia, and

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FIG. 5. A leaf of burley tobacco from a field naturally infected with Strain K. Note the mottle, and necrotic flecking towards the base of the leaf.

FIG. 6. An upper leaf of Kentucky burley 17-A naturally infected with Strain N. Strain N used in these experiments was obtained from this leaf.

FIG. 7. A plant of the Samsun variety infected with Strain N. The lower leaves have collapsed as a result of the spread of the necrosis shown in Fig. 3. Nine leaves have already died and two more died after this photograph was taken. The upper leaves were unaffected by necrosis.

FIG. 8. A burley tobacco leaf showing a common primary symptom in fields infected with etch. It is believed that this leaf was inoculated by a viruliferous insect.

zinnia. No infection was obtained on cucumber, bean, eggplant, *Vicia faba*, and sweet pea and other legumes. On tomato, Strain K caused more distortion and mottling than Strains N or V. Infection on potato was induced only with some difficulty.

Because of the simultaneous occurrence of etch, tobacco mosaic, and potato viruses X and Y in several fields, the importance of mixed infections on disease severity is being studied. Preliminary experiments show that infection with a ring spot strain of potato virus X, obtained from a naturally infected tobacco plant, combined with Strain K causes a severe disease of burley tobacco. Severe stunting, chlorosis, and "firing" results from this mixed infection, whereas much less injury is caused by either virus alone.

Symptoms Caused by Strain N

(*a*). *On Varieties Green Briar, Harrow Velvet, and Halley's Special*

Strain N is characterized mainly by the strong necrotic reaction it induces on these varieties after systemic mottling develops and at certain stages of their growth. A characteristic response of a Kentucky burley strain in the field is shown in Fig. 6. The virus strain used in these experiments was obtained from this leaf. The leaves of Kentucky 17-A burley were almost completely destroyed by this virus in the field. The first symptoms in the greenhouse appear from 10 days to two weeks after inoculation and consist of a pale green or chlorotic spot mottle, or an almost masked diffuse mottle (Fig. 4). There is little or no etching of the type occurring on burley varieties infected with Strain K. Occasionally, on rapidly growing plants, coarse, etchlike flecking and spotting occurs on some of the middle leaves. After the lower leaves have reached a certain stage of maturity, circular necrotic spots develop (Fig. 3). These spots are from 1/16 to $\frac{1}{4}$ in. in diameter and are usually surrounded by a brown border and a chlorotic zone. In the cells of this zone, large amorphous cytoplasmic inclusions may be observed. The necrosis begins on the lower leaves and moves towards the upper leaves as they mature. Necrosis often follows the main vein of the lower leaves. The bottom leaves may become entirely necrotic and collapse, whereas only a mild chlorotic mottle is visible on the upper leaves.

Fig. 9. A young leaf of Harrow Velvet burley inoculated five weeks previously with Strain K. Note the bright yellow mottle and only a trace of etching on the tip.

Fig. 10. *D. stramonium* infected with Strain K. Note the vein-clearing.

Fig. 11. *D. stramonium* infected with Strain N. This strain causes more severe distortion and rugosity than Strain K.

Fig. 12. The burley variety Green Briar naturally infected with Strain V in the field showing mottling on the upper leaves. Symptoms on the lower leaves of the same plant consisted of necrotic line patterns as shown in Fig. 14, and pronounced vein-clearing. Strain V was obtained from this plant.

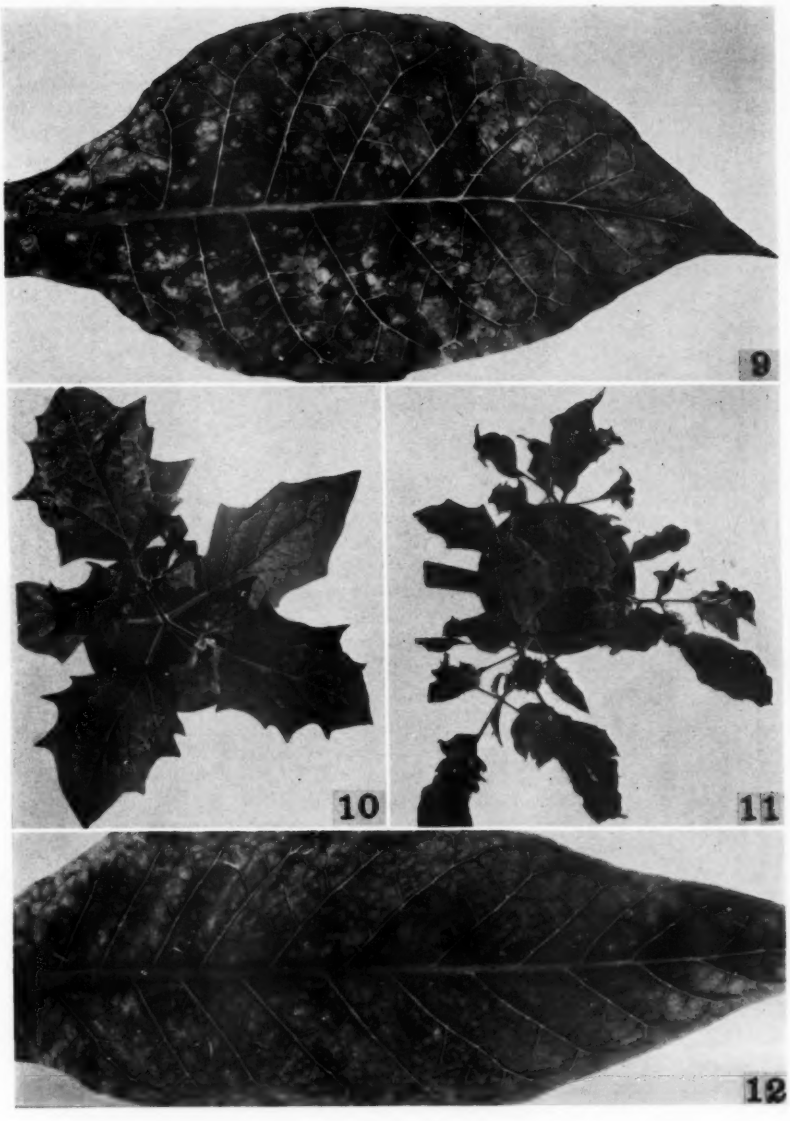
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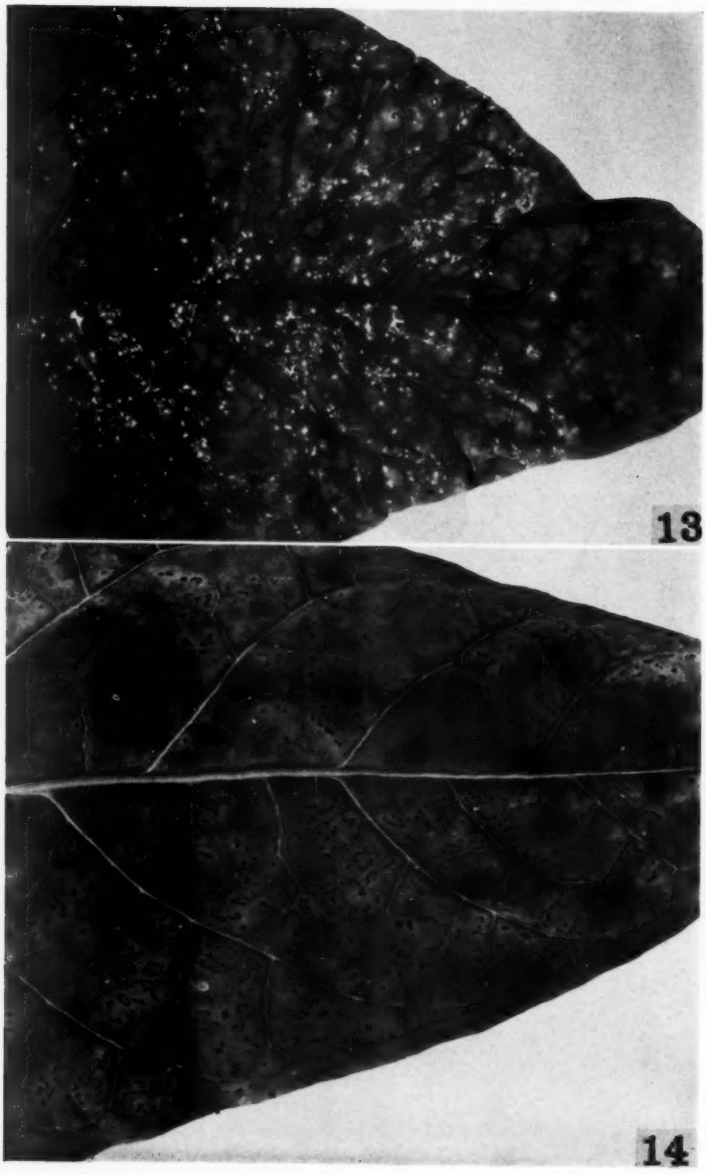
PLATE IV



(*b*). *On Variety Samsun*

The first symptoms consist of a faint chlorotic spot mottle which may be almost masked. Necrotic spotting and necrosis of the lower leaves are very severe after the plants have reached a certain stage of growth, usually about four weeks after inoculation. The leaves gradually collapse from the bottom of the plant upward (Fig. 7), although the top whorl of leaves is not affected. Necrotic flecking, similar to the etching caused by Strain K but in a much reduced form, is sometimes observed on the upper leaves.

(*c*). *On Varieties White Mammoth, Yellow Mammoth, Little Crittenden, and Delcrest*

A very faint, pale green mottle is observed on these varieties, but no necrosis and/or etching occurs on young plants in the greenhouse. On older plants, brown necrotic spots often develop as well as brown, necrotic flecks along the veins of the bottom leaves. The lower leaves become chlorotic and may wilt. The severe necrotic response of burley varieties does not occur.

(*d*). *On Datura stramonium and Pepper*

On *D. stramonium*, Strain N induces severe leaf distortion and rugosity (Fig. 11), contrasted with the milder distortion caused by Strain K (Fig. 10). Occasionally, an indistinct mottle developed on pepper but on most plants no symptoms were detected. However, a mottle sometimes developed on older, symptomless plants when these were cut back.

Symptoms Caused by Strain V

In the field, this strain caused pronounced vein-clearing with necrotic line patterns on the lower leaves of Green Briar and Harrow Velvet burley and mottling on the suckers or young leaves (Figs. 12, 14). In the greenhouse, only a mild mottle with faint vein-banding has been observed on all tobacco varieties tested, and an almost invisible, frequently masked mottle with traces of vein-banding on flue cured and dark type varieties. No necrotic reaction has been obtained in the greenhouse. A few necrotic spots, however, develop on older plants. The mottle consists of circular or diffuse, mildly chlorotic spots. A circular, mildly chlorotic spotting occurs on Samsun and on *D. stramonium*. The mottle on *Datura* may disappear and symptoms become masked. No symptoms were observed on pepper.

Fig. 13. A naturally infected field plant of the Little Crittenden variety showing necrotic spotting caused by Strain K. The chlorotic areas are not the result of virus but are indications of ripening on the mature upper leaves. A mild mottle was observed only on the suckers. No necrosis has been observed on this variety under winter greenhouse conditions. Note the similarity of these symptoms to certain physiological leaf spots.

Fig. 14. A naturally infected field plant of Green Briar (see Fig. 12) showing necrotic line patterns caused by Strain V. No necrosis has been obtained on any varieties with this strain in the greenhouse.

Summary and Discussion

It is indeed rare, if not improbable, that any virus of specific rank exists as a single strain. It is not surprising, therefore, to find at least three strains of the etch virus in Ontario. These strains differ considerably in the symptoms they cause and their behavior on different hosts (Tables I, II, III). Each strain shows a wide range of symptoms depending on the conditions of plant growth. In fact, certain symptoms observed in the field have not developed in the greenhouse under winter conditions. A significant observation on host response is the fact that tobacco varieties can be divided into two groups on the basis of their reaction to the etch virus. The varieties that respond with pronounced symptoms are termed the severe-symptom group (Table II). The inheritance of the etching reaction in *N. tabacum* is now under further investigation. The F_1 of all crosses tested thus far between severe-symptom and mild-symptom varieties have manifested mild symptoms. The F_2 have segregated into mild- and severe-symptom types. The inheritance data have not been completed and will be reported in a subsequent communication. It appears that the severe symptoms are associated with the low chlorophyll content varieties.

The differences in varietal response to etch are evident in the field as well as in the greenhouse. In the burley belt, severe-symptom (burley) and mild-symptom varieties (flue cured and dark types) are often grown on adjacent and sometimes the same farms. The most severe injury caused by this virus occurs on the former varieties, whereas the disease is usually mild or absent on the latter. All three strains of etch may be readily distinguished (Table I) on the severe-symptom varieties (Table II) of *N. tabacum*, and on *Datura*. Strain differences are not so apparent either on the mild-symptom tobacco varieties or on pepper. Strain K is similar to the "severe etch" strain previously described in the literature, whereas Strains N and V appear to differ in their failure to induce the characteristic etching symptom on the tobacco varieties tested.

In the field, symptoms caused by the etch virus are first noticed when plants are approaching the flower bud stage. Vein-clearing, often with necrotic line patterns and mottling, is the first symptom to appear. The disease becomes intensified and causes the greatest injury after the plants are topped. Symptoms appear in the absence of any mechanical operations and often coincident with the appearance of the green peach aphid (*Myzus persicae*), a vector of the virus. This insect is indicated to be the main cause of the rapid spread of the disease through the burley tobacco area in Ontario. Severely affected plants have chlorotic, "fired", and tattered upper leaves at the harvesting stage. Also, the presence of potato virus X accompanied by etch causes a still more severe disease than either virus alone. The importance of mixed infections in the virus problem in Ontario is now being studied.

The overwintering source of etch in Ontario is not known. This could be ascertained only by a survey of perennial weed hosts in the tobacco growing areas. It may be significant that etch is most prevalent in the early vegetable

growing areas, of which Leamington, Ont., is the center. Valleau and Johnson (19) have suggested that potatoes may be a reservoir of the virus. Attempts to inoculate potatoes in the greenhouse by mechanical means, however, have resulted in only a small percentage of infections. It would appear that potatoes are infected only with difficulty by the methods used, nevertheless, the role of insects as vectors of the virus on potato has not been determined. Approximately 5000 acres of early potatoes, mostly of the Irish Cobbler variety, are grown in the burley tobacco area. If only a small percentage of the potato plants were infected, the infected plants would in most instances be no great distance from tobacco fields. In any case, the importance of the potato viruses on tobacco in Ontario has become evident only since the appearance of aphids on tobacco in 1947.

Acknowledgments

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STUDIES IN THE NUTRITION OF *CORYNEBACTERIUM SEPEDONICUM* (SPIEK. AND KOTT.) SKAPT. AND BURKH.[1]

By D. S. MacLachlan[2] and F. S. Thatcher[3]

Abstract

The nutritional requirements of *Corynebacterium sepedonicum* (Spiek. and Kott.) Skapt. and Burkh. were investigated by the use of the Warburg respiration apparatus. Pantothenic acid, in a concentration of 600 micrograms (μgm.) per liter, caused the greatest growth augmentation of nine vitamins tested. The amino acids, glutamic acid, asparagine, leucine, and lysine in concentrations of 0.001, 1.0, 0.03, and 0.03 gm. per liter respectively, caused a marked increase in oxygen uptake, either individually or in combination. Yeast nucleic acid and thymonucleic acid had no significant effect on the growth rate of *C. sepedonicum*. A new medium was prepared for the isolation and maintenance of *C. sepedonicum*.

Introduction

Corynebacterium sepedonicum, the causal organism of potato ring rot, has long been noted for its slow rate of growth on synthetic media. The slow rate of growth may be due either to unfavorable physical conditions in the medium or to some very exacting nutritional requirement of the pathogen.

Burkholder (2), in 1938, prepared a medium on which he claimed that colonies of *C. sepedonicum* appeared in from three to four days. Marten *et al.* (4) found that the addition of a 1 : 1000 aqueous solution of potassium dichromate to Burkholder's medium facilitated the isolation of *C. sepedonicum* by suppressing the growth of Gram-negative bacteria. Using this modification of Burkholder's medium, these workers were able to observe colonies of the pathogen only after periods of time ranging from seven to nine days.

Skaptason (6) found that some factor or factors present in the extract from boiled potatoes had a stimulating effect on the growth of *C. sepedonicum*. He was able to replace the potato extract with a mineral solution without retarding the growth of the organism.

In preliminary experiments involving the use of 10 different isolates and Burkholder's medium, the writer was unable to obtain positive cultures in less than eight days. Consequently the present investigation of the nutritional requirements of *C. sepedonicum* was undertaken with the object of preparing a medium which would promote rapid growth of the organism.

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Starr (7), in 1948, using plate culture methods, found that biotin, nicotinic acid, and thiamine were the "active" vitamins in the nutrition of *C. sepedonicum*.

Vitamins and amino acids are required in minute quantities by bacteria, and the increase in growth caused by these materials is difficult to detect by cultural methods. In preliminary experiments in which both colony diameter and density of growth in a liquid medium were used as indices of growth, it was impossible to detect the stimulation or inhibition of growth caused by many vitamins and amino acids. In general, the results obtained by these methods lacked reproducibility. The estimation of growth by the measurement of oxygen uptake in the Warburg Respiration Apparatus was found to be much more suitable for this type of experiment and consequently this apparatus was used in the vitamin and amino acid studies.

Materials and Methods

Before an accurate nutritional study could be undertaken by the use of the Warburg apparatus, a liquid medium containing only known, chemically pure substances was essential. Several media were tested. The best results were obtained with a medium developed by Skaptason (6) which had the following composition: disodium hydrogen phosphate, 2 gm.; sodium chloride, 2 gm.; sodium citrate, 1 gm.; asparagine, 1 gm.; dextrose, 6 gm.; water, 1 liter; to this a 7 cc. aliquot of the following solution was added: $MgSO_4.7H_2O$, 30 gm.; $Fe(NH_4)(SO_4)_2.12H_2O$, 2 gm.; manganese sulphate, 2 gm.; $ZnSO_4.7H_2O$, 2 gm.; $Ca(NO)_2.4H_2O$, 2 gm.; water, 1 liter.

Aqueous solutions of the various vitamins and amino acids were prepared aseptically and stored at 15° C. in 50 ml. flasks. These solutions were not autoclaved, thus ensuring that the vitamin or amino acid was not modified or destroyed by heat.

The Warburg apparatus was of the standard type equipped with single sidearm manometer flasks. The water bath was held at a constant temperature of 28° C. Contents of the manometer flasks were as follows: basal medium, 2.0 cc.; bacterial suspension, 0.5 cc.; potassium hydroxide (10%), 0.2 cc.; test material (amino acid or vitamin) in sidearm, 0.3 cc. Thus the total content of each flask was 3 cc.

The preparation of the bacterial suspension required much care, as slight variations in procedure caused a considerable inconsistency in results. The bacterial cells were scraped from a 96-hr. slope culture of *C. sepedonicum* grown on buffered potato dextrose agar. The cells were suspended in 10 cc. of sterile *M*/50 potassium hydrogen phosphate buffer solution (pH 6.8) and centrifuged for two minutes at 3000 r.p.m. This treatment precipitated the clumps of bacteria and agar contained in the suspension without precipitating the single bacterial cells. The supernatant liquid was then decanted and the

cells were washed three times by centrifuging in fresh buffer solution, thereby ensuring that a minimum of nutrients was carried on the bacterial cells and in the suspension fluid.

Originally, 10 isolates of *C. sepedonicum* were used, but preliminary tests indicated that there was little or no difference between isolates. The results recorded are those obtained from culture C-1, isolated in 1942 by Dr. L. T. Richardson, formerly of the Division of Botany and Plant Pathology, Ottawa. This culture had been tested annually for pathogenicity to potatoes and, at the time this experiment was started, was still pathogenic after six years in culture.

A statistical analysis of the data obtained by the Warburg method indicated that a difference of ± 9 μl. of oxygen in any set of readings represented a significant increase or decrease in oxygen consumption.

All readings were taken at 30-min. intervals and, in general, the duration of each experiment was 4.5 hr.

Experimental Results

Since the various vitamins and amino acids were tested in duplicate, only four test materials could be used in each experiment. The groups of four were chosen at random, and, in general, the test material which produced the greatest growth augmentation was also used in the succeeding group of materials. Because of the difficulty of standardizing the inoculum, a comparison cannot be made between results in the different experiments.

Effect of Biotin, Pyridoxal Phosphate, and Calcium Pantothenate on the Growth of C. sepedonicum

Biotin, pyridoxal phosphate, and calcium pantothenate were added to the basal medium (Skaptason's medium) in concentrations of 1.0, 1.6, and 600 μgm. (micrograms) per liter respectively, as recommended by Gerhardt and Wilson (3). The basal medium alone was used as a check. The accumulative oxygen uptake for the various intervals is recorded in Table I.

TABLE I

THE EFFECT OF PYRIDOXAL PHOSPHATE, CALCIUM PANTOTHENATE, AND BIOTIN ON THE OXYGEN UPTAKE OF *C. sepedonicum*

Time, min.	Accumulative oxygen uptake			
	Check, μl.	Pyridoxal phosphate, μl.	Calcium pantothenate, μl.	Biotin, μl.
30	13.0	12.1	16.2	10.8
90	48.5	48.6	60.4	48.2
150	82.7	83.9	107.7	84.9
210	131.4	127.9	161.9	131.8
270	149.1	148.3	192.1	155.0

Calcium pantothenate was the only vitamin in this group that caused a significant increase in oxygen uptake. After 270 min., the oxygen uptake in the check medium was 149.1 µl. as compared with 192.1 µl. when calcium pantothenate was added (Fig. 1).

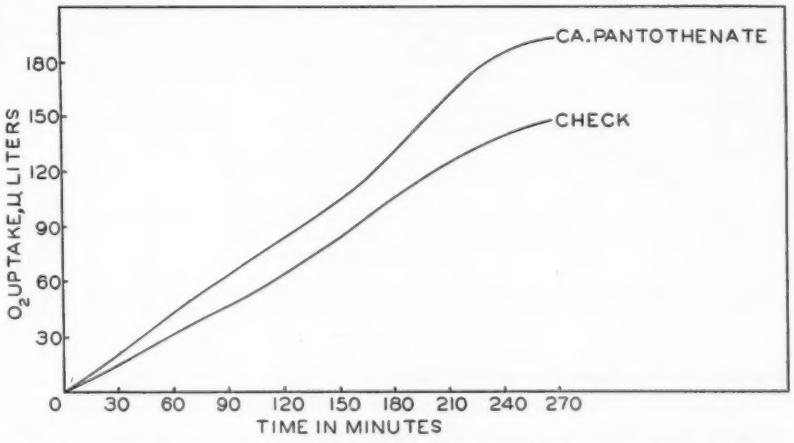

FIG. 1. The relative increase in oxygen uptake by *C. sepedonicum* obtained by adding calcium pantothenate to the basal medium at a rate of 600 µgm. per liter.

Para-aminobenzoic Acid, Thiamine, Inositol, and Calcium Pantothenate

Para-aminobenzoic acid, thiamine hydrochloride, inositol, and calcium pantothenate were added to the basal medium in concentrations of 3000, 200, 3000, and 600 µgm. per liter respectively. The results are recorded in Table II.

TABLE II

THE EFFECT OF PARA-AMINOBENZOIC ACID, THIAMINE HYDROCHLORIDE, INOSITOL, AND CALCIUM PANTOTHENATE ON THE OXYGEN UPTAKE OF *C. sepedonicum*

Time, min.	Accumulative oxygen uptake				
	Check µl.	Inositol, µl.	Calcium pantothenate, µl.	Thiamine hydrochloride, µl.	Para-amino-benzoic acid, µl.
30	21.6	10.7	19.9	12.3	16.7
90	33.7	32.0	45.9	35.5	42.2
150	60.7	72.9	92.6	71.9	85.1
210	71.7	106.7	132.5	99.1	123.1
240	81.5	127.9	155.1	116.2	142.4

All four vitamins tested in this series caused a significant increase in oxygen uptake. The greatest augmentation was caused by calcium pantothenate followed by para-aminobenzoic acid, inositol, and thiamine hydrochloride in that order (Fig. 2).

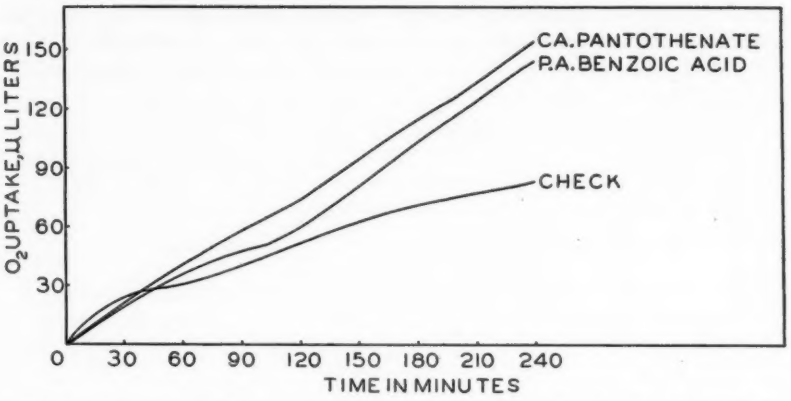

FIG. 2. A comparison of the oxygen uptake by *C. sepedonicum* in a basal medium and in media containing 3000 μgm. of para-aminobenzoic acid and 600 μgm. of calcium pantothenate.

Folic Acid, Nicotinamide, and Riboflavin

Folic acid, nicotinamide, and riboflavin were added to the basal medium in concentration of 1.0, 5000, and 1000 μgm. per liter respectively, as recommended by Umbreit, Bellamy, and Gunsalus (8). The results are recorded in Table III.

TABLE III

THE EFFECTS OF FOLIC ACID, NICOTINAMIDE, AND RIBOFLAVIN ON THE OXYGEN UPTAKE OF *C. sepedonicum*

Time, min.	Accumulative oxygen uptake			
	Check, μl.	Folic acid, μl.	Nicotinamide, μl.	Riboflavin, μl.
30	12.1	12.3	11.8	12.0
90	43.3	44.1	39.2	42.8
150	77.2	79.4	73.9	76.7
210	103.2	104.0	102.5	104.3
270	119.8	121.3	120.7	120.1

The results indicate that these three vitamins do not cause a significant increase in the oxygen uptake of *C. sepedonicum*. Nicotinamide appeared to inhibit growth slightly during the first 210 min., as the oxygen uptake was less than that recorded in the check medium.

The Effect of Certain Vitamin Combinations on the Growth Rate of C. sepedonicum

The results of the preceding experiments indicated that, in all cases, calcium pantothenate caused the greatest augmentation of growth. Inositol, thiamine, and para-aminobenzoic acid also caused an increase in the growth rate of *C. sepedonicum*, but to a lesser extent than pantothenate.

In Table IV, the effect of a combination of calcium pantothenate and each of the other three vitamins on the growth rate of *C. sepedonicum* is shown.

TABLE IV

THE EFFECT OF CERTAIN VITAMIN COMBINATIONS ON THE OXYGEN UPTAKE OF *C. sepedonicum*

Time, min.	Accumulative oxygen uptake				
	Calcium, pantothenate, µl.	Ca. pant. + inositol, µl.	Ca. pant. + *p*-aminobenzoic acid, µl.	Ca. pant. + thiamine, µl.	Check, µl.
30	16.1	16.0	15.9	16.4	12.0
90	58.3	59.0	58.5	60.2	44.3
150	107.5	108.1	109.0	106.1	79.2
210	165.7	166.9	167.5	160.5	105.7
270	215.0	210.0	216.3	200.3	118.0

These results indicate that calcium pantothenate in combination with inositol, or para-aminobenzoic acid, or thiamine, does not raise the oxygen uptake above that recorded for calcium pantothenate alone.

Further investigation showed that the optimum concentration of calcium pantothenate was in the range 600 to 700 µgm. per liter.

The Utilization of Amino Acids by *C. sepedonicum*

Certain amino acids and, more generally, specific combinations of amino acids have been shown to inhibit bacterial growth. This fact must be kept in mind in any amino acid study. Washburn and Niven (9) found an antagonistic effect between arginine and isoleucine, and between leucine and threonine in the nutrition of *Streptococcus bovis*. Small amounts of valine, and larger amounts of glutamic acid, methionine, and cystine reversed this inhibition. *Dl*-phenylalanine and *l*-tyrosine, when used separately, had no effect on growth, but, when added together, they inhibited growth.

Wyon and McLeod (10), in studies on the inhibitive action of amino acids, found that the cyclic amino acids, histidine, tyrosine, tryptophane, and phenylalanine, in small concentrations inhibited bacterial growth, whereas cystine was the most inhibitory of the chain structure amino acids.

The following experiments were planned so as to avoid the use of known inhibitory combinations of amino acids.

The Effect of Asparagine on the Oxygen Uptake of C. sepedonicum

Since the basal medium already contained 1 gm. of asparagine per liter, it was first necessary to determine the oxygen uptake of the organism in this medium as compared to the uptake in the basal medium without asparagine. The results are recorded in Table V.

TABLE V

THE EFFECT OF ASPARAGINE ON THE RATE OF OXYGEN UPTAKE OF *C. sepedonicum*

Time, min.	Accumulative oxygen uptake	
	Basal medium (no asparagine), µl.	Basal medium (asparagine), µl.
30	5.2	8.5
90	10.4	24.4
150	23.1	43.9
210	40.1	78.1
270	57.9	112.2

The results recorded in Table V indicate that asparagine greatly increases the oxygen uptake of *C. sepedonicum* (Fig. 3). The oxygen uptake in the basal

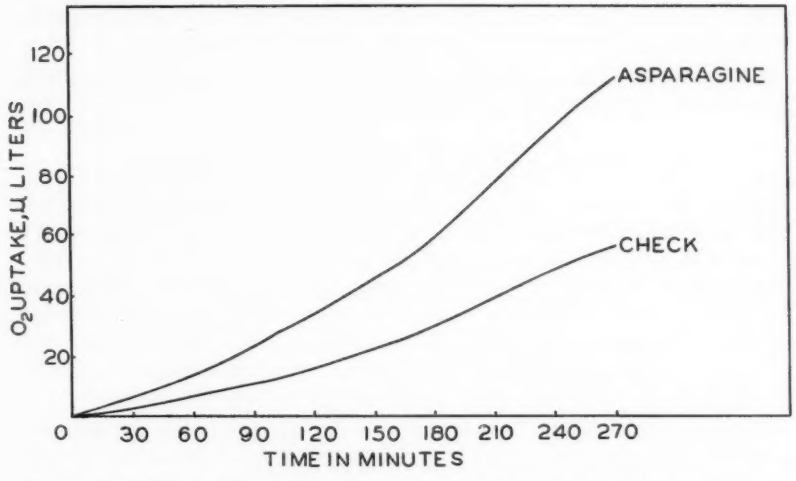

FIG. 3. A comparison between the oxygen uptake of *C. sepedonicum* in an amino-acid-free medium and in the same medium with 1 gm. of asparagine added.

medium without asparagine was almost typical of the respiration of resting cells in that, in each succeeding time interval, the oxygen uptake was almost equal to that in the preceding interval. If the cells had been multiplying, there should have been evidence of a logarithmic period in which there was a considerable increase in oxygen uptake from one interval to the next.

In the remainder of the amino acid studies, the basal medium with 1 gm. of asparagine was used as a check.

Glutamic Acid, Glycine, Cysteine, and Leucine

Glutamic acid, glycine, cysteine, and leucine were added to the basal medium in concentrations of 0.011, 0.009, 0.002, and 0.03 gm. per 100 cc. respectively. The results are recorded in Table VI.

TABLE VI

THE EFFECT OF GLUTAMIC ACID, GLYCINE, CYSTEINE, AND LEUCINE ON THE RATE OF OXYGEN UPTAKE OF *C. sepedonicum*

Time, min.	Accumulative oxygen uptake				
	Check, μl.	Glutamic acid, μl.	Glycine, μl.	Leucine, μl.	Cysteine, μl.
30	8.5	8.1	2.6	7.7	5.7
90	24.4	31.8	20.5	34.3	19.2
150	43.9	59.2	42.3	65.4	30.4
210	78.0	104.2	77.6	114.9	49.4
270	112.0	148.3	114.2	168.4	64.8

The oxygen uptake by *C. sepedonicum* was greater on media containing leucine and glutamic acid than in the basal medium (Fig. 4). Cysteine caused

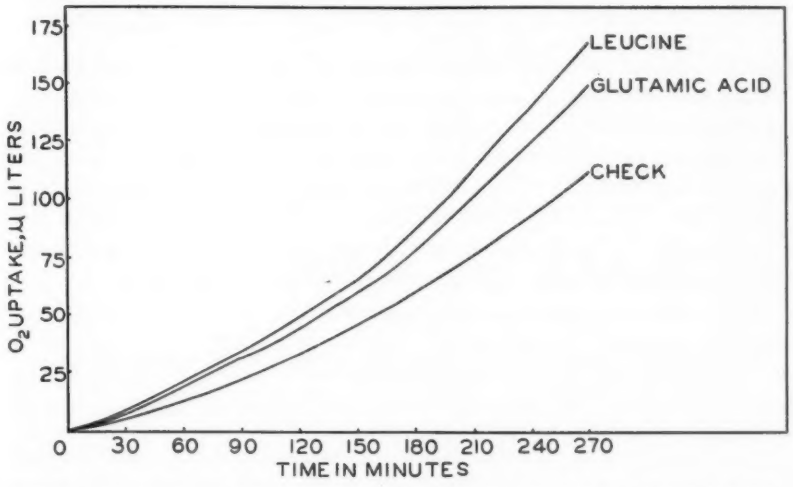

FIG. 4. A comparison of the oxygen uptake of *C. sepedonicum* in a check medium and in media containing 0.03 gm. of leucine and 0.011 gm. of glutamic acid per 100 cc.

a decrease in oxygen uptake. Reed and Orr (5) found that the addition of cysteine to a medium lowers the oxidation–reduction potential. This decrease in the rate of growth of *C. sepedonicum* when cysteine was added to the medium might, therefore, be due to the lowering of the Eh.

Alanine, Arginine, Aspartic Acid, and Lysine

Alanine, arginine, aspartic acid, and lysine were added to the basal medium in concentrations of 0.009, 0.026, 0.013, and 0.03 gm. per 100 cc. respectively. The results are found in Table VII.

TABLE VII

THE EFFECT OF ALANINE, ARGININE, ASPARTIC ACID, AND LYSINE ON THE RATE OF OXYGEN UPTAKE OF *C. sepedonicum*

Time, min.	Accumulative oxygen uptake				
	Check, µl.	Alanine, µl.	Arginine, µl.	Aspartic acid, µl.	Lysine, µl.
30	9.3	9.1	9.3	9.5	8.6
90	25.3	26.0	26.1	24.3	26.7
150	44.7	43.9	46.2	43.1	53.5
210	79.8	80.3	79.8	65.3	91.8
270	114.2	114.6	112.2	100.3	125.3

Lysine was the only amino acid in this group which caused a significant increase in the oxygen uptake of *C. sepedonicum*. Aspartic acid interfered with oxygen uptake and alanine and arginine had no significant effect.

The Effect of Combinations of Amino Acids on the Growth Rate of C. sepedonicum

Since four of the nine amino acids tested caused a significant increase in the oxygen uptake of *C. sepedonicum*, it was desirable to determine whether these amino acids, when added to the basal medium in combination, caused a greater augmentation than when added separately. The following five combinations were used. Basal medium plus (1) leucine; (2) leucine and glutamic acid; (3) leucine, glutamic acid, and lysine; (4) lysine and glutamic acid; (5) lysine and leucine. The results are recorded in Table VIII.

TABLE VIII

THE EFFECT OF A COMBINATION OF AMINO ACIDS ON THE RATE OF OXYGEN UPTAKE BY *C. sepedonicum*

Time, min.	Accumulative oxygen uptake				
	Check + (1), µl.	Check + (2), µl.	Check + (3), µl.	Check + (4), µl.	Check + (5), µl.
30	9.1	9.2	8.7	9.0	9.1
90	30.1	31.8	35.2	28.2	28.4
150	66.5	69.5	87.4	63.7	64.2
210	110.8	121.6	142.6	104.5	103.7
240	128.6	137.9	160.5	120.7	118.8

The results indicate that a combination of leucine, glutamic acid, and lysine in concentrations of 0.02, 0.11, and 0.3 gm. per 100 cc. of basal medium increased the oxygen uptake of *C. sepedonicum* more than the addition of the other combinations tested.

The Effects of Purine and Pyrimidine Bases on the Growth Rate of C. sepedonicum

Boyd, Logan, and Tytell (1) found that the addition of 50 mgm. of uracil and 87 mgm. of adenine sulphate to the medium increased the growth of *Clostridium perfringens*, a slow-growing organism.

Since calcium pantothenate augmented the growth of *C. sepedonicum*, a series of media was prepared in which pantothenate in a concentration of 600 μgm. was added in combination with uracil, adenine, guanine, and cytosine at concentrations of 50 mgm. per liter. The basal medium with pantothenate was used as a check. The results are recorded in Table IX.

TABLE IX

THE EFFECTS OF THE PURINE AND PYRIMIDINE BASES IN COMBINATION WITH CALCIUM PANTOTHENATE ON THE GROWTH RATE OF *C. sepedonicum*

Time, min.	Accumulative oxygen uptake				
	Calcium pantothenate, μl.	Ca. pant. + uracil, μl.	Ca. pant. + adenine, μl.	Ca. pant. + guanine, μl.	Ca. pant. + cytosine, μl.
30	11.1	13.9	11.0	11.1	10.9
90	54.6	42.9	55.1	53.5	53.8
150	93.1	78.0	92.8	93.5	92.9
210	123.8	105.8	124.1	123.6	123.7
270	162.4	143.7	163.5	161.7	161.9

Calcium pantothenate increased the oxygen uptake of *C. sepedonicum* as in the preceding experiments but, when uracil was added in combination with pantothenate, this action was interfered with, especially in the later stages of growth (Fig. 5). Possibly the uracil was selectively adsorbed on some surface which normally adsorbed a compound of a similar molecular configuration. Thymonucleic acid was one of the compounds commonly formed by bacteria and which contained a molecule similar to the uracil molecule. The thymonucleic acid molecule contains 5 methyl uracil in its molecular structure. This linkage might possibly be the site of competitive inhibition.

The Effect of Nucleic Acids on the Growth Rate of C. sepedonicum

Samples of both thymonucleic acid (desoxyribose nucleic acid) and yeast nucleic acid (ribose nucleic acid) were obtained. If the synthesis of thymonucleic acid is a limiting factor in the nutrition of this organism, an augmentation should result if it is added to the medium. An increase in growth should

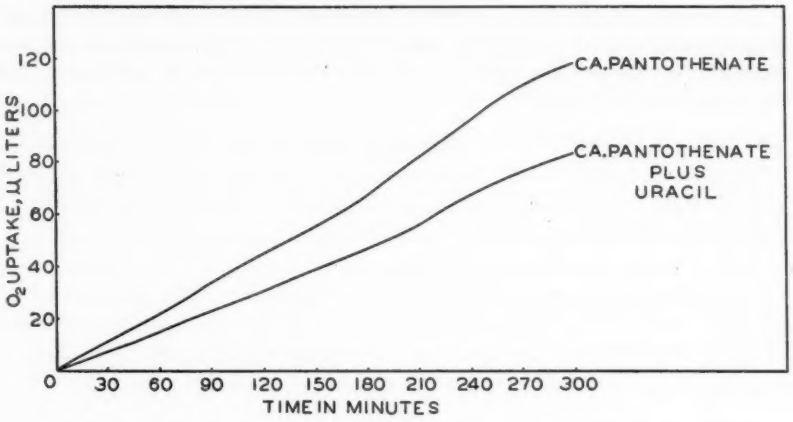

FIG. 5. The effect of a combination of uracil and calcium pantothenate on the accumulative oxygen uptake of *C. sepedonicum*.

also occur if thymine is added; and, if uracil interferes in the synthesis of thymonucleic acid by competitive inhibition with thymine, the addition of thymine along with uracil should prevent this inhibition.

Duplicate Warburg flasks were set up containing the basal medium alone, and the basal medium containing each of the following:

1. 200 mgm. thymonucleic acid per liter
2. 200 mgm. yeast nucleic acid per liter
3. 50 mgm. thymine per liter
4. 50 mgm. thymine + 50 mgm. uracil per liter

The results are recorded in Table X.

TABLE X

THE UTILIZATION OF NUCLEIC ACIDS BY *C. sepedonicum*

Time, min.	Accumulative oxygen uptake				
	Basal medium, μl.	Basal med. + thymonucleic acid, μl.	Basal med. + yeast nucleic acid, μl.	Basal med. + thymine, μl.	Basal med. + thymine + uracil, μl.
30	12.1	11.3	12.4	12.0	11.9
90	42.2	36.1	43.2	41.3	40.2
150	61.5	51.6	64.0	62.7	60.3
210	104.0	110.8	111.5	108.5	110.6
270	119.6	121.7	125.6	119.3	124.1

Neither thymonucleic acid or yeast nucleic acid caused a significant increase in oxygen uptake. The addition of thymine had no marked effect on oxygen uptake, but the addition of thymine and uracil in combination, although causing no noticeable increase in oxygen uptake, prevented the inhibition which was apparent when uracil alone was supplied. These results indicate that the synthesis of nucleic acids is not a limiting factor in the growth of *C. sepedonicum*, or that the organism is not able to utilize presynthesized nucleic acids.

Although the addition of thymine to the medium did not cause an augmentation of growth, this material was capable of neutralizing the inhibitory action of uracil by some undetermined activity. It does appear, however, that the uracil inhibition is not due to competition between uracil and thymine in the formation of thymonucleic acid.

A New Medium for the Isolation and Maintenance of *C. sepedonicum*

As a practical application of this nutritional study, a medium was prepared in which were used the various amino acids which caused an increase in the oxygen uptake of *C. sepedonicum*, as measured in the Warburg Respiration Apparatus, together with calcium pantothenate. This medium was of the following composition.

Extract of 400 gm. of potato—	
Dextrose	25.0 gm.
Agar	15.0 "
Sodium and ammonium phosphate	0.5 "
Sodium dihydrogen phosphate	0.17 "
Potassium chloride	0.20 "
Asparagine	1.0 "
Glutamic acid	0.01 "
Leucine	0.3 "
Lysine	0.3 "
Peptone	5.0 "
Calcium pantothenate	600.0 μgm.
Water	1000 cc.

Two series of dilution plates were prepared. In one series the newly developed medium was used; in the other series the medium outlined by Burkholder (2) was used. The dilutions were prepared from a 96-hr. slope culture of *C. sepedonicum* and the plates were incubated at 24° C. in a constant temperature chamber. The plates were examined daily for the appearance of colonies. The results are recorded in Table XI.

The results from Table XI show that isolations could not be made from either medium in less than six days. After a six-day incubation on the new medium, a few colonies large enough for isolation appeared, but in general, growth was characterized by a film over the surface of the medium. This same film was evident in the 1 : 10 dilution plates of Burkholder's medium after seven days.

TABLE XI

A COMPARISON OF THE GROWTH RATE OF C. *sepedonicum* ON A NEW MEDIUM CONTAINING VITAMINS AND AMINO ACIDS AND ON BURKHOLDER'S MEDIUM

Medium	Dilution	No. of days' incubation				
		5	6	7	8	9
Burkholder's medium	1 : 10	0	0	Overgrown		
	1 : 100	0	0	0	+	+
	1 : 1000	0	0	0	+	+
	1 : 10,000	0	0	0	0	0
	1 : 100,000	0	0	0	0	0
New medium	1 : 10	0	Overgrown			
	1 : 100	0	0	+	+	+
	1 : 1000	0	0	+	+	+
	1 : 10,000	0	0	0	+	+
	1 : 100,000	0	0	0	+	+

+ *indicates colonies were large enough to make transfers possible.*

After a seven-day incubation on the new medium, transfers could be made from the 1 : 100 and 1 : 1000 dilution plates (Fig. 6). Following an eight day incubation, colonies were visible on the 1 : 10,000 and 1 : 100,000 plates, but no colonies were evident on the respective plates of Burkholder's medium even after nine days.

Discussion of Results

Since *Corynebacterium sepedonicum* was able to grow in a vitamin-free medium, it must be able to synthesize all the vitamins necessary for its nutrition. However, four of the nine vitamins tested, when added separately to the medium, caused a significant increase in the growth rate of the organism. Pantothenic acid caused the greatest growth increase, and combinations of pantothenic acid, thiamine, inositol, and para-aminobenzoic acid failed to cause a greater increase in the rate of growth than the pantothenic acid alone. These results indicate that the synthesis of pantothenic acid is a factor limiting the rate of growth of *C. sepedonicum*.

The respiration of *C. sepedonicum* in an amino-acid-free medium was similar to the respiration of resting cells. This fact indicates that all the amino acids necessary for its growth cannot be synthesized by *C. sepedonicum*. Of the nine amino acids tested, asparagine caused the greatest growth augmentation, with lysine, leucine, and glutamic acid also causing significant increases in the growth rate. A combination of these four amino acids caused a greater increase in the growth rate than that recorded when they were used singly or in pairs. Thus, asparagine, lysine, leucine, and glutamic acid seem to play an important role in the nutrition of *C. sepedonicum*.

In the studies involving purine and pyrimidine bases, uracil caused a very marked inhibition of growth. An attempt was made to determine the cause of this inhibition, but no satisfactory explanation can be given at this time.

PLATE I

FIG. 6. Dilution plates (1 : 1000) of *C. sepedonicum* after a seven-day incubation.

a. Burkholder's medium.
b. New medium containing vitamins and amino acids.

Neither thymonucleic acid nor yeast nucleic acid was utilized by the organism. This result suggests either that the synthesis of nucleic acids is not a factor limiting the growth of *C. sepedonicum* or that the organism was unable to utilize these nucleic acids in the form in which they were added to the medium.

Calcium pantothenate and four amino acids were used in the preparation of a solid medium. When this medium was inoculated with a suspension of *C. sepedonicum*, colonies of a suitable size for isolation appeared after seven days of incubation. However, after several months culturing on this medium, isolations were possible in periods of time ranging from four to five days, a result which seems to indicate that the organism adapts to some extent its nutrition to suit the medium on which it is growing.

Summary

1. *C. sepedonicum* is able to grow in a vitamin-free medium.

2. Of nine vitamins tested, pantothenic acid in a concentration of 600 μgm. per liter of medium caused the greatest growth augmentation.

3. *C. sepedonicum* was unable to grow in an amino-acid-free medium.

4. A combination of the amino acids, asparagine, leucine, lysine, and glutamic acid added to the medium in concentrations of 1.0, 0.3, 0.3, and 0.11 gm. per 1000 cc. respectively, caused a greater growth augmentation than any other combination of nine amino acids tested.

5. Uracil caused a marked inhibition of growth when added to the medium.

6. Neither thymonucleic acid (desoxyribose nucleic acid) nor yeast nucleic acid (ribose nucleic acid) stimulated the growth of *C. sepedonicum*.

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A SURVEY OF HIGHER PLANTS FOR ANTIBACTERIAL SUBSTANCES[1]

By Charles J. Bishop[2] and Russell E. MacDonald[3]

Abstract

The results of a preliminary survey of 209 Nova Scotian plants are reported. In the survey five solvents: distilled water, alcohol, ether, acetone, and benzene were used to extract substances from each plant. By the use of these solvents, 33.4% of the plants tested were shown to contain one or more substances inhibitory to *Staphylococcus aureus* No. 209 and/or *Escherichia coli* A.T.C.C. No. 9637. Fifteen per cent of the extractions made showed activity against *S. aureus*, while 4.6% were active against *E. coli*. The highest incidence of activity was obtained with the ether extractions. All the plants tested are listed, and those displaying any activity are tabulated with their results. The 10 most active plants were found to be: *Cirsium arvense, Chimaphila umbellata, Eupatorium perfoliatum, Menyanthes trifoliata, Monotropa uniflora, Myrica Gale, Nymphaea odorata, Oxalis europaea, Populus gileadensis,* and *Pyrus Malus.*

Introduction

The scientific study of plants to determine their antibacterial properties is a comparatively new field of science, yet records of their medicinal uses date as far back as 4000 B.C. (13) and comments on their preparation and use appear in the writings of all early civilizations. Some of these ancient plant remedies, such as cinchona bark, have been established in modern times as possessors of antimicrobial principles that have been effectively used in combatting disease.

Apart from the findings of Penfold and Grant (18) little work of any systematic nature on the antibacterial substances possessed by higher plants was carried out until Osborn (17) made a survey of some 2300 European plants. Inspired by his results numerous other workers (1, 3, 7, 10, 16, 19) conducted surveys of a similar nature. Their results served to confirm the frequent occurrence of antibacterial substances in the flowering plants. Some workers (4, 6, 12, 15) have been able to isolate and identify the active principles which they contain. A more complete summary of this work can be found in a recent book by Florey *et al.* (9). To date all substances thus obtained have proved to be too toxic to animals for practical therapeutic use. This high incidence of toxicity has been noted also in the fungi, yet our most effective chemotherapeutics have been obtained from this group. It has been suggested (14) that some of these antibacterial substances from plants too toxic for use on animals might prove to be effective in combatting plant diseases, but as yet little has been done in this field.

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Contribution from the Department of Biology, Acadia University, Wolfville, N.S.
This project was supported by a research grant from the National Research Council of Canada.

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Materials and Methods

All plants used in this survey were collected locally. The plant material, if not tested immediately, was kept at 4° C. in a vasculum. The material could be kept fresh for at least two weeks under these conditions.

The stems, leaves, and usually the flowers and roots were macerated by means of a Waring blendor. In all cases 10 gm. of plant material was ground with 50 cc. of distilled water. The suspension was then strained through several thicknesses of cheese cloth, and 5 gm. of the strained material placed in a mortar. Five ml. of the water in which the material had been ground was added and allowed to stand with occasional grinding for 5 to 10 min. The water extract was then removed from the plant material by using the pestle as a press. To this residue 5 ml. of 95% ethyl alcohol were added and allowed to stand for 5 to 10 min. with occasional grinding. A similar procedure was followed with ethyl ether, acetone, and benzene. Owing to the volatile nature of these solvents, the plant material was not extracted for more than three minutes.

The above liquid extracts were tested for antibacterial activity using the method of assay suggested by Heatley (11) for penicillin. Control tests carried out at intervals during the survey showed that the solvents used had no inhibitory effect on the bacteria. The test organisms were *Staphylococcus aureus* No. 209 (Department of National Health and Welfare, Ottawa) and *Escherichia coli* A.T.C.C. No. 9637. In the case of positive or doubtful results each extract was retested.

In some cases it was difficult to determine the exact extent of inhibition owing to poor growth of the test organism or to other factors. This difficulty was generally eliminated by covering the surface of the agar (after incubation) with a solution of mercuric chloride. This caused the colonies to become opaque and white, thus defining them against a dark background.

Results

During the summer and fall of 1950, 209 species of plants representing 65 families were collected and tested for antibacterial content. In the classification of these plants Gray's Manual of Botany (8th edition) (8) was regarded as final authority for identification and nomenclature. Of the 209 plants tested 33.4% were found to be active to some degree. Nine hundred and forty extractions were made, of which 146 (15%) were active against *S. aureus* and 44 (4.6%) against *E. coli*. Ten per cent of the water extracts, 19.1% of the alcohol extracts, 23.3% of the ether extracts, 20.5% of the acetone extracts, and 14.6% of the benzene extracts were active against one or both of the test organisms. Benzene extracts were made on only 109 of the plants tested.

In the test of each extract the degree of activity was determined from the diameter (measured in millimeters) of the zone of inhibition. A list of all the plants tested in this survey is given in Table I, and all the plants which yielded active extracts are shown in Table II with their recorded degree of activity.

TABLE I

LIST OF PLANTS TESTED FOR ANTIBACTERIAL ACTIVITY

(THOSE MARKED WITH AN ASTERISK SHOWED INHIBITION (SEE TABLE II))

Aceraceae
Acer saccharum Marsh.

Alismataceae
**Sagittaria cuneata* Sheldon

Amaranthaceae
Amaranthus retroflexus L.

Apocynaceae
**Apocynum androsaemifolium* L.
A. sibiricum Jacq.

Aquifoliaceae
Ilex verticillata (L.) Gray

Araceae
Arisaema Stewardsonii Britt.

Araliaceae
Aralia hispida Vent.
**A. nudicaulis* L.

Asclepiadaceae
Asclepias incarnata L.

Boraginaceae
Mertensia maritima (L.) S. F. Gray.

Caprifoliaceae
**Diervilla Lonicera* Mill.
**Linnaea borealis* L. var. *americana* (Forbes) Rehd.
Sambucus pubens Michx.

Caryophyllaceae
Lychnis alba Mill.
L. chalcedonica L.
Stellaria graminea L.
Saponaria officinalis L.

Chenopodiaceae
Atriplex patula L.
Chenopodium album L.
Salicornia europaea L.
Suaeda maritima (L.) Dumort.

Commelinaceae
Tradescantia virginiana L.

Compositae
**Achillea Millefolium* L.
**Ambrosia artemisiifolia* L.
**Anaphalis margaritacea* (L.) C. B. Clarke
Aster Novi-belgii L.
A. cordifolius L.

Compositae—*Concluded*
**A. foliaceus* L.
A. acuminatus Michx.
**Bidens frondosa* L.
**B. cernua* L.
Cichorium Intybus L.
**Cirsium arvense* (L.) Scop.
**C. vulgare* (Savi) Tenore
Coreopsis lanceolata L.
**Erechtites hieracifolia* (L.) Raf.
**Erigeron philadelphicus* L.
**Eupatorium maculatum* L.
**E. perfoliatum* L.
Gnaphalium uliginosum L.
**G. Macounii* Greene
**Hieracium aurantiacum* L.
H. paniculata L.
H. Pilosella L.
**H. pratense* Tausch.
Iva frutescens L. var. *oraria* (Bartlett) Fern. & Grisc.
**Inula Helenium* L.
Lactuca canadensis L.
Matricaria matricarioides (Less.) Porter
M. maritima L. var. *agrestris* (Knaf) Wilmott
Prenanthes trifoliolata (Cass.) Fern.
**Rudbeckia laciniata* L.
Solidago bicolor L.
**S. canadensis* L.
**S. flexicaulis* L.
S. graminifolia (L.) Salisb.
**S. sempervirens* L.
Sonchus arvensis L.
S. asper (L.) Hill
Tanacetum vulgare L.
Taraxacum officinale Weber
Tragopogon pratensis L.
Tussilago Farfara L.

Cornaceae
**Cornus canadensis* L.
C. stolonifera Michx.

Corylaceae
Alnus crispas (Ait.) Pursh var. *mollis* Fern.
Corylus cornuta Marsh.

Crassulaceae
Sedum purpureum (L.) Link.

Cruciferae
**Alyssum saxatile* L.
Cakile edentula (Bigel.) Hook.
**Lepidium densiflorum* Schrad.
Raphanus Raphanistrum L.
**Sisymbrium altissimum* L.
S. officinale (L.) Scop.

TABLE I—*Continued*

LIST OF PLANTS TESTED FOR ANTIBACTERIAL ACTIVITY—*Continued*

(THOSE MARKED WITH AN ASTERISK SHOWED INHIBITION (SEE TABLE II))

Cucurbitaceae
Echinocystis lobata (Michx.) T. & G.

Empetraceae
**Empetrum atropurpureum* Fern. & Wieg.
**E. nigrum* L.

Equisetaceae
Equisetum arvense L.
E. hyemale L. var. *affine* (Engelm.) A. A. Eaton

Ericaceae
**Andromeda glaucophylla* Link.
Epigea repens L. var. *glabrifolia* Fern.
Gaultheria procumbens L.
**Kalmia angustifolia* L.
K. polifolia Wang.
Ledum groenlandicum Oeder
**Rhododendron canadense* (L.) Torr.
**Vaccinium angustifolium* Ait. var. *laevifolium* House

Euphorbiaceae
Euphorbia Cyparissias L.
E. Helioscopia L.
**E. vermiculata* Raf.

Fagaceae
Quercus alba L.

Gentianaceae
**Menyanthes trifoliata* L. var. *minor* Raf.

Guttiferae
**Hypericum perforatum* L.

Hamamelidaceae
Hamamelis virginiana L.

Hippocastanaceae
Aesculus Hippocastanum L.

Iridaceae
Iris versicolor L.

Juglandaceae
**Juglans cinerea* L.

Labiatae
Galeopsis Tetrahit L. var. *bifida* (Boenn.) Lej. & Court.
Glechoma hederacea L. var. *micrantha* Moricand
Hedeoma pulegioides (L.) Pers.
Leonurus Cardiaca L.
Mentha arvensis L.
Origanum vulgare L.

Labiatae—*Concluded*
Prunella vulgaris L.
**Scutellaria lateriflora* L.
S. epilobiifolia A. Hamilton

Leguminosae
**Amphicarpa bracteata* (L.) Fern.
**Apios americana* Medic.
Lathyrus japonicus Willd.
**Lupinus polyphyllus* Lindl.
**Medicago sativa* L.
M. lupulina L.
**Melilotus alba* Desr.
Robinia Pseudo-Acacia L.
Trifolium arvense L.
Vicia Cracca L.

Liliaceae
Convallaria majalis L.
**Smilacina racemosa* (L). Desf.
Trillium erectum L.

Malvaceae
Malva moschata L.
Althaea rosea Cav.

Myricaceae
Comptonia peregrina (L.) Coult.
**Myrica Gale* L.
**M. pensylvanica* Loisel.

Nymphaeaceae
**Nymphaea odorata* Ait.

Oleaceae
**Syringa vulgaris* L.

Onagraceae
Epilobium angustifolium L.
E. glandulosum Lehm. var. *adenocaulon* (Haussk.) Fern.
Oenothera perennis L.
Oenothera sp.

Orchidaceae
Habenaria lacera (Michx.) Lodd.
**H. psycodes* (L.) Spreng.
Spiranthes cernua (L.) Richard

Orobanchaceae
Epifagus virginiana (L.) Bart.

Oxalidaceae
**Oxalis europaea* Jord.
O. montana Raf.

Papaveraceae
Papaver orientale L.

TABLE I—*Concluded*

LIST OF PLANTS TESTED FOR ANTIBACTERIAL ACTIVITY—*Concluded*

(THOSE MARKED WITH AN ASTERISK SHOWED INHIBITION (SEE TABLE II))

Pinaceae
**Abies balsamea* (L.) Mill.
Juniperus communis (L.) var. *depressa* Pursh.
Tsuga canadensis (L.) Carr.

Plantaginaceae
Plantago lanceolata L.
**P. juncoides* Lam. var. *decipiens* (Barn.) Fern.
P. major L.

Plumbaginaceae
Limonium Nashii Small

Pontederiaceae
Pontederia cordata L.

Polygonaceae
Fagopyrum sagittatum Gilib.
Polygonum convolvulus L.
P. cuspidatum Sieb. & Zucc.
P. exsertum Small.
P. Persicaria L.
P. scabrum Moench.
**Rumex Acetosella* L.
R. obtusifolius L.

Portulacaceae
**Portulaca oleracea* L.

Primulaceae
Lysimachia punctata L.
L. Nummularia L.

Pyrolaceae
**Chimaphila umbellata* (L.) Bart. var. *cisantlantica* Blake
Monotropa Hypopithys L.
**M. uniflora* L.
Pyrola elliptica Nutt.

Ranunculaceae
Anemone canadensis L.
A. virginiana L.
Aquilegia vulgaris L.
Coptis groenlandica (Oeder) Fern.
Clematis virginiana L.
**Ranunculus acris* L.
**Thalictrum polygamum* Muhl.

Rosaceae
Amelanchier laevis Wieg.
Geum canadense Jacq.
Potentilla anserina L.
**P. argentea* L.
P. simplex Michx.
P. tridentata Ait.
**Pyrus Aucuparia* (L.) Gaertn.

Rosaceae—*Concluded*
P. floribunda Lindl.
**P. Malus* L.
Sanguisorba minor Scop.
**Spiraea latifolia* (Ait.) Borkh.
S. tomentosa L.
Rosa borbonica Morr.

Rubiaceae
Galium asprellum Michx.
G. erectum Huds.
Mitchella repens L.

Salicaceae
Populus alba L.
**P. balsamifera* L.
**P. gileadensis* Rouleau
**P.* sp.

Sarraceniaceae
Sarracenia purpurea L.

Saxifragaceae
Ribes sativum Syme

Scrophulariaceae
Euphrasia americana Wettst.
**Chelone glabra* L.
Linaria vulgaris Hill.
Rhinanthus Crista-galli L. var. *fallax* (Wimm. & Grab.) Druce
Verbascum Thapsus L.
**Veronica officinalis* L.

Solanaceae
Solanum Dulcamara L.

Thymelaeaceae
Daphne Mezereum L.

Tiliaceae
Tilia americana L.
T. platyphyllos Scop.

Typhaceae
Typha angustifolia L.
T. latifolia L.

Umbelliferae
Daucus Carota L.
Hydrocotyle americana L.

Violaceae
Viola nephrophylla Greene

Vitaceae
Parthenocissus quinquefolia (L.) Planch.

Zosteraceae
Potamogeton natans L.

TABLE II

PLANTS WHOSE EXTRACTS SHOWED ANTIBACTERIAL ACTIVITY

Plant	*S. aureus*					*E. coli*				
	1*	2	3	4	5	1*	2	3	4	5
Alismataceae										
Sagittaria cuneata	—	—	12	—	—	—	—	—	—	—
Apocynaceae										
Apocynum androsaemifolium	—	—	—	—	—	—	—	18	18	18
Araliaceae										
Aralia nudicaulis	—	14	—	—	—	—	—	—	—	—
Caprifoliaceae										
Diervilla Lonicera	12	16	14	—	—	—	—	—	—	—
Linnaea borealis var. *americana*	13	—	—	13	—	—	—	—	—	—
Compositae										
Achillea Millefolium	15	—	15	—	x	—	—	—	—	x
Ambrosia artemisiifolia	12	12	—	—	x	12	12	—	—	x
Anaphalis margaritacea	—	15	18	—	x	—	—	—	—	x
Aster foliaceus	—	—	—	—	—	—	—	—	—	14
Bidens frondosa	—	—	18	12	—	—	—	—	—	—
B. cernua	—	13	13	13	—	—	—	—	—	—
Cirsium arvense	—	18	17	14	—	—	PI	PI	16	20
C. vulgare	—	14	18	16	—	—	—	—	—	—
Erechtites hieracifolia	—	—	12	12	—	—	—	—	—	—
Erigeron philadelphicus	—	—	—	15	x	—	—	—	12	x
Eupatorium maculatum	—	—	16	—	—	—	—	—	—	—
E. perfoliatum	16	12	23	17	—	—	—	—	—	—
Gnaphalium Macounii	—	16	23	19	—	—	—	—	—	—
Hieracium aurantiacum	—	—	22	—	x	—	—	—	—	x
H. pratense	—	—	—	14	x	—	12	—	—	x
Inula Helenium	—	PI	16	—	—	—	—	15	—	—
Rudbeckia laciniata	—	16	—	13	16	—	—	—	—	—
Solidago canadensis	—	20	—	—	—	—	20	15	—	—
S. flexicaulis	—	—	—	15	—	—	—	—	—	—
S. sempervirens	—	10	—	—	—	—	—	—	—	—
Cornaceae										
Cornus canadensis	—	—	17	17	—	PI	—	—	—	—
Cruciferae										
Alyssum saxatile	—	11	—	—	x	—	—	22	—	x
Lepidium densiflorum	—	—	13	10	x	—	—	—	—	x
Sisymbrium altissimum	11	—	11	—	x	—	—	—	—	x
Empetraceae										
Empetrum atropurpureum	—	14	16	—	—	—	—	—	—	—
E. nigrum	—	14	12	14	—	—	—	—	—	—

NOTE: P.I. = *Partially inhibited.*
x = *No extract made.*
*1 = *Water extract.*
2 = *Alcohol extract.*
3 = *Ether extract.*
4 = *Acetone extract.*
5 = *Benzene extract.*

TABLE II—*Continued*

PLANTS WHOSE EXTRACTS SHOWED ANTIBACTERIAL ACTIVITY—*Continued*

Plant	*S. aureus*					*E. coli*				
	1*	2	3	4	5	1*	2	3	4	5
Ericaceae										
Andromeda glaucophylla	—	—	—	15	—	—	—	—	—	—
Kalmia angustifolia	17	—	14	—	x	19	23	20	27	x
Rhododendron canadense	—	—	16	14	x	—	18	20	18	x
Vaccinium angustifolium var. *laevifolium*	12	12	—	—	—	20	14	17	18	14
Euphorbiaceae										
Euphorbia vermiculata	—	—	—	15	—	—	—	—	—	—
Gentianaceae										
Menyanthes trifoliata var. *minor*	19	19	20	20	21	—	—	—	—	—
Guttiferae										
Hypericum perforatum	—	12	14	—	x	—	—	—	—	x
Juglandaceae										
Juglans cinerea	—	—	15	—	14	—	—	—	—	—
Labiatae										
Scutellaria lateriflora	—	12	14	18	—	—	—	—	—	—
Leguminosae										
Amphicarpa bracteata	—	—	11	—	12	—	—	—	—	—
Apios americana	—	14	19	13	17	—	—	—	—	—
Lupinus polyphyllus	—	11	—	11	x	—	—	—	—	x
Medicago sativa	—	—	—	—	—	—	—	16	—	—
Melilotus alba	—	—	13	12	x	—	—	15	—	x
Liliaceae										
Smilacina racemosa	—	—	—	12	16	—	—	—	—	—
Myricaceae										
Myrica Gale	25	29	23	20	x	—	—	—	—	x
M. pensylvanica	11	—	—	—	x	12	—	—	—	x
Nymphaeaceae										
Nymphaea odorata	18	16	16	18	—	—	—	15	13	—
Oleaceae										
Syringa vulgaris	10	10	PI	10	x	—	—	—	—	x
Orchidaceae										
Habenaria psycodes	15	15	—	—	x	—	—	—	—	x
Oxalidaceae										
Oxalis europaea	12	—	—	15	x	15	12	12	12	x
Pinaceae										
Abies balsamea	—	13	18	—	—	—	—	—	—	—
Plantaginaceae										
Plantago juncoides var. *decipiens*	—	12	—	—	—	—	—	—	—	—
Polygonaceae										
Rumex Acetosella	—	—	12	—	x	—	—	—	—	x
Portulacaceae										
Portulaca oleracea	—	—	—	—	—	—	—	15	—	—

TABLE II—*Concluded*

PLANTS WHOSE EXTRACTS SHOWED ANTIBACTERIAL ACTIVITY—*Concluded*

Plant	*S. aureus*					*E. coli*				
	1*	2	3	4	5	1*	2	3	4	5
Pyrolaceae										
Chimaphila umbellata var. *cisantlantica*	—	15	27	15	15	—	—	15	—	—
Monotropa uniflora	20	21	17	13	x	—	—	—	—	x
Ranunculaceae										
Ranunculus acris	—	—	14	—	x	—	—	15	13	x
Thalictrum polygamum	—	—	—	20	17	—	—	—	—	—
Rosaceae										
Potentilla argentea	PI	—	—	17	x	—	—	—	—	x
Pyrus Aucuparia	20	20	—	23	—	—	—	—	—	—
P. Malus	—	20	24	22	18	—	—	—	—	—
Spiraea latifolia	—	13	13	14	15	—	—	12	12	—
Salicaceae										
Populus balsamifera	—	26	17	17	14	—	—	—	—	—
P. gileadensis	15	29	22	20	22	—	10	—	—	—
P. sp.	—	18	16	16	11	—	—	—	—	—
Scrophulariaceae										
Chelone glabra	18	—	—	—	—	—	—	—	—	—
Veronica officinalis	—	—	13	15	x	—	—	—	—	x

Discussion

In this survey a higher percentage of antibacterial activity was found among the plants tested than has been reported in any previous survey. This is considered to be due to the wider range of solvents used, since the percentage of activity obtained with water extracts correlates favorably with other surveys which used water only as a solvent. In each test the potential strength of the antibacterial substance is not necessarily shown accurately by the zone of inhibition. Because its concentration in the extract is unknown, its true activity cannot be definitely determined. Furthermore, there is no evidence that the potency of the substance is correlated with a wide range of solubility. However, the desirability of using several solvents is clearly indicated, for if water alone had been used, over half of the plants would have been classified as possessing no antibacterial substance.

While in general the results of this survey correlate favorably with those of other surveys of a similar nature, extracts of the Ranunculaceae did not give the high activity reported in the family by Osborn (17), nor did those of the Cruciferae. One probable reason for this is a difference in techniques used in the surveys. This, however, would not seem to account for it entirely, and possibly an alternative explanation is that variations in the season, time of day, and habitat may have an important influence on the content of the antibacterial substance. Such variations are known to occur in the alkaloid content of certain plants.

The use of solvents other than water brings up the possibility that dissolved chlorophyll may be the active substance. However, Carlson *et al.* (5) could find no evidence that the chlorophyll present had any effect on the growth of the test organisms, and in our survey many of the plants whose extracts appeared to contain large quantities of chlorophyll, were inactive against both of the bacteria used.

In many cases the extracts of certain plants were noted to cause stimulation rather than inhibition of the test organisms. Boas (2) was one of the first to call attention to this phenomenon and suggested that the presence of a stimulating substance may cover up or greatly lessen the effect of any inhibitor present. It may be, however, that these stimulatory substances are actually antibacterial compounds in high dilutions, as such stimulation has been found to occur in the case of many of the known chemotherapeutics.

Further work is being carried out at present in the isolation of the active principle in the leaves of *Pyrus Malus* (apple). A number of the other plants, e.g., *Myrica Gale*, *Vaccinium* sp., *Menyanthes trifoliata*, and certain species of *Populus* offer equally fruitful possibilities.

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INHIBITION OF PLANT PATHOGENIC BACTERIA *IN VITRO* BY ANTIBIOTICS AND QUATERNARY AMMONIUM COMPOUNDS[1]

By H. Katznelson and M. D. Sutton

Abstract

The bacteriostatic effect of certain antibiotics and quaternary ammonium compounds on representative genera and species of phytopathogenic bacteria has been studied. For *Xanthomonas* species aureomycin was the most potent agent with terramycin and polymyxin next; 0.1 to 0.05 p.p.m. aureomycin completely inhibited growth of most of the cultures at 24 hr. Terramycin was the most effective compound against *Pseudomonas* species followed by streptomycin and aureomycin; 0.2 to 0.1 p.p.m. terramycin inhibited growth of every culture tested with one exception. For *Corynebacterium* species aureomycin was most effective with neomycin and terramycin next, the activity of aureomycin varying from 0.4 to 0.05 p.p.m. Chloromycetin was the least effective of the antibiotics tested in this series of experiments. A comparison of the relative sensitivity of the three genera to a given antibiotic revealed that aureomycin and polymyxin were most effective on *Xanthomonas*, terramycin and streptomycin on *Pseudomonas*, and neomycin and chloromycetin on *Corynebacterium*. *Erwinia amylovora* was most sensitive to streptomycin and *E. carotovora* to polymyxin. The latter organism was generally more resistant to the antibiotics than the former. A number of other antibiotics and quaternaries: streptothricin, clavacin, gliotoxin, aspergillic acid, tyrothricin, penicillin, Roccal, Hyamine 1622, and compound L4-669 were also tested against strains of *X. phaseoli* and *P. phaseolicola* (agents of common and halo blight respectively of beans). None of these was as effective as aureomycin, polymyxin, streptomycin, or terramycin, the activity of the quaternaries being similar on the whole to that of chloromycetin.

Introduction

The treatment of many diseases of man and animal with antibiotics has become established practice. During the past few years there has been increasing interest also in the possible application of these substances in the control of diseases of plants. Most of the known antibiotics have already been tested against many phytopathogenic fungi and bacteria and a number are being studied more extensively for practical disease control (11, 32, 36, 38).

The present communication concerns the inhibition of bacterial plant pathogens *in vitro* by certain common antibiotics (especially those active against Gram-negative bacteria) as a preliminary step to the practical application of these substances. Since the literature on this subject is widely dispersed in various journals which are not related to plant pathology it was considered advisable to review it here.

Many of the studies on antibiosis and phytopathogenic bacteria deal with the antagonistic effect of various microorganisms in pure culture or in complex systems, such as soil, on these bacteria (6, 12, 18, 21, 26, 27, 29). The availability of pure antibiotics during the past decade has permitted studies of their specific bacteriostatic and bactericidal effects on plant pathogenic bacteria. Many of the known antibiotics have been tested although the

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results vary considerably depending on methods of testing, nature of the antibiotic, conditions of growth, and nature of the test organism and consequently comparisons of effectiveness are very difficult. Among the most comprehensive of these studies in regard both to number of pathogens and antibiotics tested are those of Waksman *et al.* (33) and Gilliver (19). The former, employing both agar-cup diffusion and agar-streak dilution methods tested six substances against nine representative plant pathogenic bacteria (including Gram-positive and negative species). They found that these organisms did not behave as a group, some being more sensitive to certain agents and others more resistant. *Phytomonas glycinea,** which is Gram-negative, was more sensitive to actinomycin (which has a high activity on Gram-positive bacteria) than *P. stewarti* and equal in sensitivity to the Gram-positive *P. michiganensis*. The activity of streptothricin ranged from 500 dilution units for *P. solanacearum* to 150,000 for *P. tumefaciens*. Fumigacin and chaetomin, active chiefly on Gram-positive bacteria, were active against some of the pathogens but not on others, whereas clavacin showed little difference against these bacteria. Penicillin has relatively little effect on Gram-negative organisms, although certain of the plant pathogens (*P. tumefaciens*) were somewhat sensitive. By means of the agar diffusion technique, they demonstrated that the plant pathogens fell midway between *Escherichia coli* and *Staphylococcus aureus* and *Bacillus subtilis* in sensitivity. The bactericidal action of the antibiotics on *P. tumefaciens* was very similar to that on *S. aureus* and nearly comparable to their bacteriostatic action.

Gilliver (19) tested 14 antibiotic substances on representative species of plant pathogens by the dilution method and found generally that Gram-positive bacteria were more sensitive than Gram-negative forms to proactinomycin, mycophenolic acid, helvolic acid, penicillin, tyrothricin, and gliotoxin, the highest titers being obtained with the last three. *Xanthomonas malvacearum* gave the highest titer with penicillic acid (1 : 1,280,000), but otherwise Gram-positive and Gram-negative bacteria were equally sensitive to penicillic acid, aspergillic acid, and clavacin. *Xanthomonas* species were the most sensitive of the Gram-negative bacteria.

Most of the other studies on antibiotics and plant pathogenic bacteria are restricted to tests of a single substance on one or more species of these organisms. Waksman and Woodruff (35) reported complete inhibition of *Erwinia carotovora* by 100 mgm. actinomycin per liter and partial inhibition with 10 mgm. Anderson (2) exposed 20 species of bacterial pathogens to filtrates of *Aspergillus clavatus* and found that the lethal dose ranged from 2 – 6% and the bacteriostatic dose from 1 – 4%; *Bacterium stewarti* and *Xanthomonas pruni* were the most sensitive. Penicillin was not effective against the latter organisms. Johnson *et al.* (24) showed that 10 species of *Phytomonas*, including *michiganensis*, were inhibited by 1 : 10^3 of gliotoxin and delayed in growth by lower dilutions. *P. michiganensis* was completely inhibited at a dilution of 1 : 10^5 after 24 hr. Penicillin was reported to inhibit *Erwinia carnegieana*

* *Species names are those used by the authors under discussion.*

on agar, with indications of inhibition of *Corynebacterium sepedonicum* as well (8, 9). *Phytomonas pruni* was sensitive to streptomycin at 0.25μ per ml. and to neomycin at 0.1μ per ml. (34). Tomatin stopped growth of *Phytomonas solanacearum* on agar but penicillin had no effect (23).

In an extensive study on mycophenolic acid, Florey *et al.* (17) showed it to be effective against *C. michiganense* at 1 : 320,000 and on *C. sepedonicum at* 1 : 160,000 (both Gram-positive organisms) but not effective even at 1 : 5000 on various other (Gram-negative) species of bacterial plant pathogens. Rudolph (30) found that certain strains of *Erwinia amylovora* were inhibited by penicillin but not others. *X. juglandis* was more sensitive than *E. amylovora*. The antibiotic was also bactericidal. Ark (4, 5) reported inhibition of representative species of phytopathogens, both Gram-positive and Gram-negative, by streptomycin. *X. translucens* is sensitive to subtilin (20) and to various antibiotics from higher plants (10, 31). De Ropp (14) made a thorough study of the effect of seven antibiotics on six strains of *Phytomonas tumefaciens* by the dilution method. Aureomycin, chloromycetin, streptomycin, and streptothricin were the most active; wide variation among strains was observed.

Bacteriostatic Effect of Antibiotics

All tests were conducted with nutrient broth except those for the corynebacteria, in which case yeast extract, glucose, and inorganic salts were added to the broth. Concentrated stock solutions of the antibiotics were prepared in sterile distilled water or in the appropriate solvent and diluted in the culture medium, as described elsewhere (25). All dilutions were based on weights of the active substances rather than their salts. Inoculum consisted of 24-hr. fluid cultures grown in 50 ml. Erlenmeyer flasks; one 3 mm. loopful was used per tube. Readings were made for complete, partial, and no inhibition at 24 and 48 hr.; only those for complete inhibition at 24 hr. are summarized in Tables I, II, III, and IV.

Aureomycin stands out as the most potent antibiotic against *Xanthomonas* species, with terramycin, polymyxin, streptomycin, neomycin, and chloromycetin in order of decreasing effectiveness. For *Pseudomonas* species, the order of potency is terramycin, streptomycin, aureomycin, polymyxin, neomycin, and chloromycetin, whereas, for the Gram-positive *Corynebacterium* species, aureomycin is again superior, followed by neomycin, streptomycin, terramycin, and chloromycetin. Polymyxin, active on Gram-negative bacteria primarily, is only weakly effective on the corynebacteria. Streptomycin was the most active against *E. amylovora* and chloromycetin the least. *E. carotovora* was apparently somewhat more resistant to antibiotics than *E. amylovora* and the most susceptible to polymyxin. On the whole, it appears that aureomycin and polymyxin are the most effective against the xanthomonads, terramycin and streptomycin against the pseudomonads, and neomycin and to some extent chloromycetin against the corynebacteria.

TABLE I

INHIBITION OF *Xanthomonas* SPECIES*

Organism	Antibiotics and quaternary ammonium compound						
	Aureomycin	Chloromycetin	Neomycin	Polymyxin	Streptomycin	Terramycin	Roccal
X. beticola	102.4	1.6	6.4	3.2	1.2	51.2	0.8
X. campestris (1)**	102.4	3.2	3.2	102.4	3.2	102.4	6.4
X. " (2)	204.8	—	—	—	—	—	—
X. juglandis	102.4	3.2	3.2	51.2	19.2	6.4	3.2
X. malvacearum	102.4	6.4	12.8	25.6	38.4	12.8	3.2
X. phaseoli (1)	102.4	6.4	6.4	51.2	19.2	25.6	1.6
X. " (2)	102.4	6.4	3.2	51.2	9.6	25.6	3.2
X. " var. *fuscans* (1)	102.4	6.4	12.8	25.6	9.6	25.6	3.2
X. " " " (2)	204.8	—	12.8	102.4	19.2	51.2	3.2
X. " " *sojensis*	204.8	6.4	6.4	51.2	9.6	51.2	1.6
X. pruni (1)	204.8	6.4	0.8	25.6	9.6	51.2	1.6
X. " (2)	204.8	3.2	6.4	51.2	19.2	51.2	1.6
X. " (3)	102.4	6.4	6.4	51.2	6.4	102.4	3.2
X. vesicatoria (1)	102.4	3.2	1.6	1.6	2.4	6.4	1.6
X. " (2)	204.8	6.4	—	—	—	—	—
X. " (3)	102.4	3.2	3.2	25.6	9.6	25.6	3.2
X. translucens f. sp. *cerealis*	204.8	12.8	6.4	51.2	19.2	51.2	3.2
X. cerealis	204.8	3.2	6.4	25.6	6.4	51.2	3.2
X. hordei	204.8	6.4	3.2	25.6	6.4	51.2	3.2
X. phleipratensis	204.8	6.4	6.4	25.6	12.8	51.2	6.4
X. secalis	204.8	12.8	6.4	6.4	12.8	102.4	6.4
X. undulosa	204.8	6.4	3.2	1.6	6.4	51.2	3.2

* *Expressed as reciprocals of dilutions in hundred thousands causing complete inhibition of growth.*

** *Figures in parentheses represent strains tested.*

TABLE II

INHIBITION OF *Pseudomonas* SPECIES*

Organism	Antibiotics and quaternary ammonium compound						
	Aureomycin	Chloromycetin	Neomycin	Polymyxin	Streptomycin	Terramycin	Roccal
P. angulata	25.6	3.2	3.2	6.4	19.2	51.2	0.8
P. atrofaciens	51.2	1.6	12.8	12.8	39.2	51.2	0.4
P. coronafaciens (1)	102.4	3.2	25.6	25.6	156.8	409.6	0.8
P. " (2)	102.4	1.6	25.6	25.6	156.8	102.4	0.4
P. glycinea (1)	12.8	0.8	12.8	12.8	76.8	12.8	0.8
P. " (2)	25.6	3.2	25.6	25.6	51.2	102.4	3.2
P. lachrymans	51.2	3.2	6.4	12.8	25.6	102.4	0.4
P. phaseolicola (1)	51.2	3.2	12.8	25.6	76.8	102.4	0.8
P. " (2)	25.6	1.6	25.6	1.6	51.2	102.4	0.8
P. pisi (1)	51.2	1.6	12.8	12.8	102.4	102.4	0.8
P. pisi (2)	25.6	3.2	12.8	25.6	51.2	51.2	0.8
P. syringae (1)	102.4	1.6	12.8	6.4	102.4	51.2	0.4
P. " (2)	25.6	1.6	6.4	12.8	25.6	51.2	0.4
P. tabaci	25.6	1.6	6.4	12.8	76.8	51.2	0.8

* *See footnote, Table I.*

TABLE III

INHIBITION OF *Corynebacterium* AND *Erwinia* SPECIES*

Organism	Antibiotics and quaternary ammonium compound						
	Aureomycin	Chloromycetin	Neomycin	Polymyxin	Streptomycin	Terramycin	Roccal
C. fascians	25.6	3.2	51.2	0.4	6.4	12.8	3.2
C. flaccumfaciens (1)	51.2	12.8	51.2	0.8	76.8	6.4	3.2
C. " (2)	51.2	1.6	25.6	0.4	51.2	6.4	3.2
C. " (3)	102.4	3.2	25.6	6.4	6.4	102.4	6.4
C. insidiosum	102.4	6.4	51.2	0.4	12.8	25.6	12.8
C. michiganense	51.2	6.4	6.4	0.4	6.4	6.4	3.2
C. poinsettiae	25.6	0.8	6.4	0.4	6.4	3.2	3.2
C. sepedonicum (1)**	204.8	6.4	102.4	1.6	51.2	25.6	3.2
C. " (2)	204.8	12.8	102.4	0.8	51.2	25.6	3.2
C. " (3)	102.4	6.4	102.4	0.8	51.2	12.8	6.4
E. carotovora	12.8	3.2	25.6	51.2	12.8	12.8	—
E. amylovora	51.2	3.2	51.2	51.2	204.8	51.2	—

* *See footnote, Table I.*
** *C. sepedonicum read at 48 hr.*

A comparison of species and strain sensitivities among the xanthomonads reveals no marked differences with aureomycin. *X. beticola* was somewhat more resistant to chloromycetin, polymyxin, and streptomycin than the remaining species. *X. pruni* Strain 1 was more resistant to neomycin than the other two strains, and *X. vesicatoria* Strain 1 was among the most resistant to neomycin, polymyxin, streptomycin, and terramycin. *X. undulosa* and *X. secalis* were among the most resistant to polymyxin.

P. coronafaciens was perhaps the most sensitive of the pseudomonads to most of the substances used, particularly aureomycin and streptomycin. *P. glycinea* Strain 1 was among the most resistant to aureomycin, chloromycetin, and terramycin. *P. phaseolicola* Strain 1 was the most resistant to polymyxin, and *P. angulata* to streptomycin. *C. sepedonicum* strains were in general more sensitive to antibiotics than the majority of the other corynebacteria and *C. poinsettiae* among the most resistant. Considerable variation in sensitivity of strains of *C. flaccumfaciens* may be noted.

As little or no work has been done with these antibiotics on plant pathogenic bacteria it is not possible to make comparisons. Waksman *et al.* (34) reported that *Phytomonas pruni* was inhibited by streptomycin, 0.25μ per ml. and neomycin 0.1μ per ml., amounts which are somewhat smaller than those shown to inhibit this organism in Table I. Ark (5), working with streptomycin, reported "antibiotic activity as demonstrated by a filter paper disc method is equally strong against many different types of phytopathogens belonging to the genera *Erwinia*, *Xanthomonas*, *Corynebacterium*, *Pseudomonas*, and *Agrobacterium*". However the examples given as illustration in his paper do not appear to support this general conclusion (compare *A. tumefaciens*

TABLE IV

INHIBITION OF STRAINS OF *Xanthomonas phaseoli* AND *Pseudomonas phaseolicola* BY ANTIBIOTICS AND QUATERNARY AMMONIUM COMPOUNDS*

Organism	Aureomycin	Chloromycetin	Polymyxin	Streptomycin	Streptothricin	Clavacin	Gliotoxin	Aspergillic acid	Tyrothricin	Hyamine 1622	QAC L4-669	Roccal	Penicillin
X. phaseoli													
(*a*)	204.8	6.4	51.2	6.4	0.8	3.2	1.6	1.6	3.2	1.6	1.6	12.8	0.8
(*b*)	204.8	6.4	51.2	6.4	1.6	6.4	1.6	1.6	1.6	1.6	3.2	12.8	1.6
(*c*)	204.8	6.4	51.2	6.4	0.8	3.2	1.6	1.6	3.2	1.6	3.2	6.4	0.8
(*d*)	204.8	12.8	25.6	6.4	1.6	6.4	3.2	1.6	12.8	1.6	3.2	6.4	1.6
(*e*)	204.8	12.8	51.2	6.4	0.8	3.2	1.6	1.6	3.2	1.6	1.6	12.8	0.4
(*f*)	204.8	12.8	51.2	6.4	1.6	3.2	1.6	0.8	1.6	1.6	3.2	6.4	1.6
(*g*)	204.8	6.4	51.2	12.8	1.6	6.4	1.6	1.6	3.2	1.6	3.2	6.4	0.4
P. phaseolicola													
(*a*)	102.4	3.2	12.8	102.4	0.8	12.8	—	0.8	0.8	0.8	0.8	3.2	0.2
(*b*)	—	—	—	—	—	—	—	—	—	0.8	0.8	3.2	0.2

* *See footnote, Table I.*

with the remaining organisms, Page 609). The results given in Tables I, II, and III show that pseudomonads are distinctly more sensitive to this antibiotic than xanthomonads and generally more sensitive than the corynebacteria. For the species given in these tables, few of the antibiotics tested by Gilliver (19), Waksman *et al.* (33), and others attain the inhibitory levels of aureomycin, polymyxin, terramycin, streptomycin, or neomycin. Certain exceptions are actinomycin and penicillin for *X. phaseoli*, *P. glycinea*, *X. pruni*, and *C. michiganense* (33); tyrothricin and gliotoxin for *C. sepedonicum* and *C. michiganense;* and penicillic acid for *X. malvacearum* (19).

A comparison of the inhibitory concentrations of the antibiotics used in the present study with those for nonphytopathogenic Gram-negative bacteria, as given by Alexander *et al.* (1), may be of interest. Streptomycin appears to be more active against the phytopathogenic pseudomonads and polymyxin and aureomycin against both these and the xanthomonads. Chloromycetin (1, 28) is equally effective against phyto- and nonphytopathogenic forms. Neomycin (34) exhibits the same order of activity for most of the Gram-negative bacteria, whereas terramycin (15, 22) is somewhat more active against the plant pathogens. The sensitivity of the phytopathogenic corynebacteria is on the whole greater than that of *C. diphtheriae* to neomycin (34) and aureomycin (16). *C. sepedonicum* and *C. flaccumfaciens* are also more sensitive to streptomycin than *C. diphtheriae*.

Since interest in this work originated in attempts to control common blight (*X. phaseoli*) and a number of strains of the organism were available, tests were also conducted with these, a number of additional antibiotics being used as well (Table IV). The results again show aureomycin to be the most potent substance studied, with polymyxin next. None of the remaining antibiotics was particularly effective and strain variation was not pronounced, except possibly Strain *d* with tyrothricin. None of the antibiotics tested by Waksman *et al.* (33) against *X. phaseoli* compares in potency with aureomycin or polymyxin, although actinomycin approaches the potency of either. Ark (5) showed distinct inhibition of *X. phaseoli* with streptomycin at 1 : 100,000, and slight at 1 : 1,000,000.

Bacteriostatic Effect of Quaternary Ammonium Compounds

The use of quaternary ammoniums in plant pathology has been very limited. They have been tried as seed disinfectants (7) and to a slight extent on certain bacterial plant pathogens (13). A number of these substances were tested against *X. phaseoli* and *P. phaseolicola* strains (Table IV) and the most effective one was then tested on the remaining species (Tables I, II, and III). The data in Table IV show that Roccal was the most effective of the three compounds tested. *P. phaseolicola* was somewhat less sensitive than *X. phaseoli*, the effect of Roccal being of the same order as that of chloromycetin. The inhibitory concentrations of Roccal, as given in Tables I, II, and III, are somewhat lower for these two species than the concentrations given in Table IV. However, it may be seen that this compound was less

effective than the antibiotics against both the pseudomonads and the xanthomonads, but about of the same order of activity as chloromycetin for the corynebacteria. Again, it was least active against the pseudomonads.

Discussion

Of the antibiotics shown to be effective against phytopathogenic bacteria, aureomycin and terramycin are generally outstanding; streptomycin, neomycin, and polymyxin are also very effective though somewhat more specific, e.g. streptomycin against the pseudomonads, and neomycin on the corynebacteria. The availability of most of these substances, especially aureomycin, terramycin, and streptomycin, is particularly encouraging in regard to the practical control of plant diseases. Their solubility in water is an added advantage with respect to treatment of internally infected seed by soaking. However, before they can be applied in practice, it must be shown that they are not phytotoxic, as actidione and streptomycin have been shown to be (3, 37). Another difficulty, inherent in soaking procedures in general, is the possible encouragement of saprophytic and pathogenic fungi in or on the seed during soaking and drying and the possible spread of disease in the soaking solution from a few infected seed to the healthy seed. These are problems which must be studied; evidence that they can be overcome is already available (5, 36).

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STUDIES OF CANADIAN THELEPHORACEAE

VIII. *CORTICIUM GALACTINUM* (FR.) BURT[1]

By L. T. White[2]

Abstract

During the course of an investigation into the decays of white pine in certain regions of Ontario it was found that the fungus, *Corticium galactinum* (Fr.) Burt, was important as an organism destroying considerable woody material in the roots and butts of living trees. Because of the increasing interest in this species, the early history and the literature concerning the pathogenicity on roots of apple trees as well as other hardwood and coniferous species has been reviewed. A revised description, based on the study of a portion of the type specimen from the Fries herbarium and a comparison with specimens from apple and coniferous species, is presented. The taxonomy and relationships of *C. galactinum* are discussed. Detailed comparison is made with *C. odoratum* (Fr.) B. & G. and a description of that species is included. Cultural studies show that *C. galactinum* is heterothallic and of the tetrapolar type of interfertility and may possess multiple allelomorphs at the loci responsible for heterothallism.

Introduction

During the course of an investigation into the decays of white pine in certain regions of Ontario (White (36)), it became evident that some of the organisms causing decay had received little attention. Recent studies indicate that one of these, *Corticium galactinum* (Fr.) Burt, may occur commonly as an organism responsible for root and butt rots in several coniferous species. Interest in this fungus as a potential cause of considerable loss in lumber and pulpwood has been increasing. There is, however, little published information concerning the biology of *C. galactinum* or its pathogenicity in this association.

C. galactinum has been known as a parasite of living apple trees for many years. Von Schrenk in 1902 (31) first reported *Thelephora galactina* Fr. as a widely distributed parasite of apple trees in West Virginia, Kentucky, Southern Illinois, Arkansas, and Oklahoma, and as the cause of considerable loss to apple growers in that area during the preceding 30 years. The fungus appeared as a root rot of apple trees in orchards set out on newly cleared land. Von Schrenk made the suggestion that the pathogen was transferred from nearby oak trees to young apple trees, killing the latter within a year. Identification of the causal organism was made by Dr. E. A. Burt. Von Schrenk and Spaulding (32) reported in 1909 that this fungus was a common root parasite of oak trees in various parts of the Ozark Mountains and spread

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Contribution from the Department of Botany, University of Toronto, Toronto, Ont., and Contribution No. 1090 from the Division of Botany and Plant Pathology, Science Service, Department of Agriculture, Ottawa, Canada. Based on part of a thesis presented in April, 1950, to the University of Toronto in partial fulfilment of the requirements of the degree of Doctor of Philosophy.

Previous numbers in this series were published in Can. J. Research, C, 26 : 129-139; 143-157. 1948: in C, 27 : 147-156; 241-252. 1949: and in C, 28 : 63-77; 525-534; 716-725. 1950.

[2] *Formerly Graduate Student and Demonstrator, Department of Botany, University of Toronto, now Forest Pathologist, Dominion Laboratory of Forest Pathology, Toronto, Ont.*

thence to fruit trees on recently cleared land. Archer (1) in 1927 made brief mention that "living roots of apple trees were attacked by *C. galactinum*" in a report concerning diseases of fruit and nut crops in the United States.

Except for these brief early reports no reference to this disease was found in the literature until 1940. At that time Cooley and Davidson (13) published an account of their observations on *C. galactinum* as an active parasite of apple trees. Their studies were begun in 1932 and dealt with the occurrence of this disease in Virginia, Maryland, Tennessee, Delaware, and Indiana, in orchards planted on newly claimed lands. In 1948 Cooley (12) reviewed the observations made during 15 years. These observations revealed that *C. galactinum* had been responsible for the death of many apple trees in infected orchards. It is stated that infection is dependent upon the presence of woody material in the soil. The fungus is able to live saprophytically on stumps and other debris in the soil; sporulating on such material to provide inoculum for later infection of the apple trees. Other plants listed by these investigators as susceptible to this disease are species of *Rubus*, *Cornus*, *Rhus*, *Lychnis*, as well as certain ornamentals such as *Kalmia* and *Ilex*.*

The pathogenicity of *C. galactinum* on *Malus* and certain other hardwood species, as noted above, has been known for some time. The importance of this fungus as the causal organism in root and butt rot of conifers has only become evident in recent years (Figs. 11, 12). In 1948 Marshall (22), in connection with investigations into the cause of white pine needle blight in Connecticut, reported that many trees so affected possessed root infections caused by *C. galactinum*. Decay studies directed towards forest management, which are currently being conducted in various parts of Canada, are demonstrating the presence of this fungus as a root-rot producing organism in many coniferous species. Although the detailed results of these studies have not yet been published it has become evident that, in addition to parasitism of white pine, *C. galactinum* may be held responsible for decay in living firs (*Abies balsamea* (L.) Mill. and *A. lasiocarpa* (Hook.) Nutt.) and in western white cedar (*Thuja occidentalis* (L.)). Waldie (35) has recorded the importance of this species as a decay-causing organism in spruce in British Columbia (*Picea glauca* (Moench) Voss var. *albertiana* (S. Brown) Sarg. and *P. engelmanni* Parry). Nordin (29) states that preliminary investigations indicate that *C. galactinum* causes important losses in *Abies balsamea* in northern New Brunswick. It is likely that more coniferous species will be added to this list as investigations into decays of conifers proceed.

Further, it is possible that, in addition to causing losses in the valuable lumber at the butts of living conifers, the fungus may be important as a cause of mortality among the advance reproduction of these species under forest

** The following reference has appeared since the present contribution was in press: Cooley, J. S. Control of white root rot with chloropicrin in a border of ornamental plants. Phytopathology, 41 : 379-380. 1951. In this paper Cooley reports a serious outbreak of root rot due to C. galactinum in a planting of ornamental shrubs and herbaceous perennials on "made" land at Silver Springs, Maryland. Observations indicate that white root rot may attack a wide range of ornamental shrubs and herbaceous perennials. Successful treatment of localized infection areas with chloropicrin for control of the disease is indicated.*

stands. Observations have indicated that young trees in older white pine stands may become infected through their root systems. Examination of 20 balsam fir seedlings of 2 in. D.B.H.,* chosen at random in an acre sample plot in the Timagami Lake area, revealed that the roots and basal parts of all seedlings were badly decayed. Infection may have occurred through root grafting with roots of infected trees or through wounded roots though no detailed studies have yet been made. Cultures from decayed wood established that this root and butt rot was caused by *C. galactinum*. The evidence strongly suggests that this fungus may be responsible for a high mortality among advanced reproduction.

The importance of *C. galactinum* as an organism causing rot in frondose species of importance to the forester is not known. Few studies have been conducted and there is a general lack of knowledge concerning decay organisms of hardwood forest trees, particularly in Canada. However, *C. galactinum* is commonly encountered as a saprophyte on hardwood material and it may be that further investigation will reveal its importance as a root rot of hardwoods in the forest.

The original identification of *C. galactinum* as an organism responsible for root and butt rots in forest trees was made by Dr. M. K. Nobles. It is apparent that the cultural characteristics of *C. galactinum* show a marked similarity to those of *Poria subacida* (Peck) Sacc. In her treatise on the identification of cultures of wood-rotting fungi (28), Dr. Nobles has pointed out that key patterns of *P. subacida* coincide with those for several other species; and indeed, the key patterns for *C. galactinum* which were kindly made available to the writer also show a close conformity with those for *P. subacida*. It is possible that, in the past, some of the rot attributed to *P. subacida* may have been caused by *C. galactinum*. Dr. Nobles reports, however, that in the light of her present knowledge confusion no longer exists. The main diagnostic character for cultures of *C. galactinum* is the presence of oil-filled hyphal tips, usually with minute pointed structures along the sides which exude droplets of oil. These structures and the completely bleached agar distinguish *C. galactinum* from all other species the cultural characters of which have already been determined.

Because of the increasing evidence of the importance of the species as an organism responsible for significant losses through root and butt rot in both broad-leafed and coniferous trees, and since little work had been done on the organism itself, a special study of certain phases of the biology of *C. galactinum* was undertaken.

Corticium galactinum

The original description of *Corticium galactinum* (Fr.) Burt appeared in a paper by Elias Fries (14) dealing with fungi collected in Denmark. The specimen on which the description was based was collected by H. W. Ravenel

* *D.B.H. – diameter measured at breast height.*

in South Carolina and communicated to Fries by M. A. Curtis. The description of this North American species appears as an addendum to the account of Danish fungi and reads as follows:

> "5. *Thelephora galactina*, resupinata, late effusa, incrustans, colliculosa glabra, lactea, ambitu indeterminate,
>
> Ad terram juxta fossas – Curtis 1601
>
> Crustam sistit late et indeterminate effusam cum arena omnino concrescentum, in vivo mollem in siccis induratum et contiguam more generes. Haec ad *Th.* pallidas Americanas v.c. *Th. candidam*, *pallidam* etc. accedit, ut *Th. crustacea* et *caesia* ad Europaeas fuscas."

The collector's data with reference to the substratum on the label of the type specimen in the Fries Herbarium reads: "In radicibus ad lateram fossarum", and implies an association with roots which was not referred to by Fries in his description of the new species. The inscription on Curtis's portion of the type at the Farlow Herbarium reads simply ". . . . . ad latera fossarum". The portion of the type collection in the Fries Herbarium at Uppsala which was examined was obviously on soil. It was made available through the courtesy of Dr. John Eriksson.

Certain errors and omissions have occurred in previous descriptions of this fungus. Gloeocystidia have not been previously described. The description that follows is based upon a portion of the type specimen, supplemented by material collected in Northern Ontario and samples of the fungus occurring on apple roots and *Cornus* loaned by Dr. R. W. Davidson.

Corticium galactinum (Fr.) Burt, Missouri Bot. Gard. Ann. 13 : 199. 1926; Cooley and Davidson, Phytopath. 30 : 139. 1940; Cooley, Phytopath. 38 : 110. 1948.

? *Thelephora alnea* Fr., Syst. Mycol. 1 : 446. 1821.

? *Stereum alneum* Fr., Epicr. Syst. Mycol. p. 554. 1838.

? *Thelephora suaveolens* Fr. Elenchus Fung. 1 : 208. 1828.

? *Stereum suaveolens* Fr. Epicr. Syst. Mycol. p. 553. 1838.

Thelephora galactina Fr., F. Soc. Sci. Upsal. Acta III, 1 : 136, 1851; Sacc. Syll. Fung. 6 : 541, 1888; von Schrenk, Bot. Gaz. 34 : 65. 1902.

Corticium odoratum var. *alni* (Fr.) sensu Bres., Ann. Mycol. 18 : 63, 1920; Bourdot and Galzin, Hyménomycètes de France, p. 225. 1928.

Fructifications (Figs. 10 and 13) when dry white to cream-colored or ochraceous, when moist and sporulating possessing a waxy appearance and ranging in color from cream to ochraceous, even, adnate, closely and indeterminately effused, thinning at the margin, perennial, varying in thickness with age to 500 μ or more, in section two-year or older specimens exhibiting a slight zonation when stained with phloxine; subiculum coriaceous, mostly composed of fibrous, sparsely branched, nonseptate hyphae which are hyaline to slightly yellow in color and 1.5 – 3.5 μ in diameter, closely compacted

and with thick refractive walls, becoming reddish-brown in Melzer's solution, extending to the surface of the fructification; upper hyphae in part thin-walled with clamp connections; gloeocystidia flexuous, elongate-cylindrical $40-120 \times 20-50\ \mu$, nearly colorless or with fragmented refractive contents, variable in frequency, interspersed in the subiculum and attaining the surface, occasionally projecting slightly beyond the level of the hymenium; hymenium weakly developed, basidia at first obpyriform, becoming long cylindrical, flexuous or ventricose-clavate $20-35 \times 2-3.5\ \mu$, emerging beyond the surface to $5\ \mu$, bearing four slender, straight sterigmata $4-5\ \mu$ in length; basidiospores broadly elipsoid $4-5.5 \times 1.5-3\ \mu$, hyaline, flattened slightly on one side with prominent lateral apiculus, walls thin, smooth, nonamyloid.

Occurring on roots of living *Malus*, *Rubus*, *Salix*, and *Cornus*, and broadly effused on rotting logs of *Quercus*, *Betula*, *Tilia*, and other frondose species and on coniferous wood including *Pinus*, *Picea*, *Abies*, *Tsuga*, and *Thuja*. Sporophores on roots and logs may occasionally grow out over surrounding soil and debris to fruit in abundance.

Distribution: Canada to Texas and westward to the Pacific Coast; Europe; the West Indies; and Japan. Common in northern North America.

Specimens examined:*

Ontario: on *Abies balsamea*, Lake of Two Rivers, Algonquin Park, June 24, 1939, H. S. Jackson, *14258*; Camp 8, Lake Timagami, T.F.R., Aug. 19, 1948, L. T. White, *23341*; on *Betula*, Timagami Island, Lake Timagami, T.F.R., Aug. 10, 1931, H.S.J., *5033*, (confirmed by Litschauer and Rogers); on *Picea mariana*, south of Raith, Thunder Bay District, Sept. 13, 1944, R. F. Cain, *19154*; on *Picea*, Loon Lake Portage, Lake Timagami, T.F.R., Aug. 23, 1946, R.F.C., *21046*, NY; on *Pinus strobus*, Camp 8, Lake Timagami, T.F.R., Aug. 24, 1948, *23342*; Aug. 25, 1948, *23343*; on *Pinus resinosa*, Wilno, July 24, 1948, M. K. Nobles, *23337*, OTB *21129*; *23339*, OTB *21132*; on *Populus*, Petawawa Forest Experiment Station, Chalk River, Sept. 5, 1947, H.S.J., *22489*; on *Prunus*, Algonquin Park, June 8, 1939, H.S.J., *14239*; on *Quercus*, Petawawa Forest Experiment Station, Chalk River, Sept. 5, 1947, H.S.J., *22490*; on *Thuja occidentalis*, Camp 8, Lake Timagami, T.F.R., Aug. 19, 1948, L.T.W., *23340*; Sept. 1, 1948, L.T.W., *23338*; on coniferous wood, Skunk Lake Portage, Lake Timagami, T.F.R., Sept. 1, 1933, R.F.C., *5680* (confirmed by Litschauer); Mono Mills, May 24, 1939, H.S.J., *14410*; on deciduous wood, south of Aurora, July 12, 1946, H.S.J., *20571*, NY.

Quebec: on *Abies balsamea*, Lac Jaune, Duchesnay, Aug. 24, 1938, R. F. Cain, *11238*; Pine River, near Duchesnay, Aug. 26, 1938, H. S. Jackson; on dead wood, near Fairy Lake, Hull, Oct. 17, 1902, John Macoun 86; on old *Fomes*, Duchesnay, July 26, 1938, H.S.J.

* *All collections listed below are represented in the Mycological Herbarium of the University of Toronto, TRT. The numbers of the Ontario collections are those of that herbarium. Specimens from other regions are for the most part divisions of collections from other herbaria and the abbreviations used are as proposed in Chronica Botanica, 5 : 142-150. 1939, to which should be added VFP = Dominion Laboratory of Forest Pathology, Victoria, B.C. In some cases herbarium numbers cited are those of the collector.*

British Columbia: on *Abies grandis*, Gillespie Road, V.I., June 28, 1948, H. S. Jackson; on *Alnus rubra*, Mount Douglas Park, Saanich, V.I., Aug. 9, 1946, P. J. Salisbury, VFP *2394*, OTB *17016*; East Sooke Road, V.I., June 29, 1948, H.S.J.; on *Picea canadensis*, Aleza Lake, Sept. 9, 1945, V. J. Nordin, VFP *2238*; July 13, 1948, J. R. Tannhauser, VFP *4472*; on conifer, Robert's Creek, Aug. 4, 1932, I. Mounce, OTB *F6063*; Royston, July 29, 1938, I. Mounce, OTB *F8977*; East Sooke Road, June 29, 1948, H.S.J.; West Saanich Road, V.I., June 29, 1948, H.S.J.

Maine: on *Picea rubra*, Codyville, June 11, 1934, H. G. Eno, BPI *FP81138*; on red spruce stump, near Greenville, Sept. 21, 1949, R. W. Davidson, BPI *FP94412*; on *Thuja occidentalis*, Indian Township, June 11, 1934, H.G.E., BPI *FP81281*; on conifer stump, Greenville, Sept. 22, 1949, R. W. Davidson et al., BPI *FP94410*.

New Hampshire: on *Abies balsamea*, Carrol, July 23, 1932, J. R. Hansbrough, BPI *FP81319*; May 30, 1934, H. G. Eno, BPI *FP81126*, UPS; on *Betula lutea*, Carrol, May 21, 1936, H.G.E., BPI *FP81255*; Lincoln, Sept. 28, 1939, H.G.E., BPI *FP84882*, on *Picea rubra* log, Waterville, Aug. 19, 1937, H.G.E., BPI *FP82320*; on *Pinus* log, North Conway, May 15, 1918, L. O. Overholts *5082*; on *Tsuga canadensis*, Plainfield, June 18, 1936, H.G.E., BPI *FP81394*.

Vermont: on *Tsuga canadensis*, Mt. Tabor, May 6, 1936, H. G. Eno, BPI *FP81392*.

New York: on hardwood, McLean Bogs, near Ithaca, July 14, 1934, W. L. White *1408*; on bark of conifer, Seventh Lake near Inlet, Sept. 12 and 13, 1935, A. M. and D. P. Rogers, Ex. Herb. D.P.R. (apud typum det.).

Connecticut: on *Carpinus caroliniana*, N. Bloomfield, June 3, 1934, H. G. Eno, BPI *FP81282*.

Michigan: on rotten log in alder, cherry, and maple thicket, Vermillion, July 20, 1914, A. H. Povah, MICH; on white pine, New Richmond, Nov. 11, 1916, E. W. Hartwell, MICH; on *Tilia americana* Leonard, Aug. 23, 1950, H. S. Jackson; on hardwood, Ann Arbor, July 28, 1932, C. A. Brown *480*, MICH.

Washington: on conifer log, Lake Wilderness, Sept. 11, 1915, C. H. Kauffman, MICH.; on deciduous wood, Lake Wilderness, Sept. 15, 1915, C.H.K., MICH.

Virginia: on *Malus*, 10 miles from Leesburg, Oct. 19, 1936, Davidson, Cooley and Crandall, BPI *FP71383*; Middleton, summer 1937, J. S. Cooley, BPI *FP86200*.

South Carolina: in radicibus ad latera fossarum, H. W. Ravenel, comm. M. A. Curtis *1601*, **type,** FH, UPS.

Tennessee: on stump, Mont de Conte, June 22, 1944, L. R. Hesler, TENN *16405*.

Florida: Eustis Lake, Jan. 10, 1891, L. M. Underwood *9*, NYS; on hardwood, Gainsville, March 26, 1938, W. A. Murrill *F16139*.

California: on *Pseudotsuga*, Bishop Pine Lodge, Trinidad, Feb., 1948, H. E. Parks *7181*.

Czechoslovakia: on *Abies alba*, near Trebušany, Aug. 1934, A. Pilát, PR: near Trebušany, Aug. 1938, A. Pilát, PR, UPS.

Poland: on *Alnus*, Polonia rossica, 1901, Eichler (as *Stereum alneum* Fr., genuinum) ex herb. Bresadola, herb. Burt, FH.

Finland: auf *Pinus*, Mustialia, Sept. 29, 1880. Karsten, ex von Höhnel herb., FH (as *Stereum odoratum* Fr.).

Basidia appear to develop during damp weather of summer and fall but soon collapse in drier weather. Mature basidia are frequently difficult to demonstrate on collections made during periods of drought. Fruit bodies which were used to obtain single spore cultures yielded few basidia after being dried.

As noted above *Corticium galactinum* was originally described by Fries under the name *Thelephora galactina*. Burt (11) in his monograph of *Corticium* listed the species as *Corticium galactinum* (Fr.) Burt and cited Moffat, Chicago Acad. Sci. Bull. 7 : 137 : 1909 as the place and date for the transfer to the genus *Corticium*. The reference by Moffat in a paper dealing with the Hymenomycetes in the Chicago area, however, cannot be considered as other than an incidental mention since Burt's name did not appear and there is no evidence to substantiate a valid transfer from the genus *Thelephora* to *Corticium* at that time. The authentic transfer to the genus *Corticium* appears to occur first in the monograph of Burt (11) where the combination is cited with authority and synonomy and accompanied by a lengthy description.

Burt (11) included *Corticium rigescens* B. & C. as a doubtful synonym of *C. galactinum*. The species is based on four collections, with different numbers (186, 187, 118, 191), all presumably made by Fendler in Venezuela. In the Berkeley and Curtis collection at the Farlow herbarium there are two packets with four pieces representing three of the numbers cited by Cooke in the original description. These are numbers 186, 188, and 191. Number 187 is not represented and 188 may be the correct number for the 118 cited by Cooke. The three collections represent three different species, none of which is in the best of condition. Only No. 191 shows any characters indicative of possible relationship with *C. galactinum*. That specimen does have somewhat comparable gloeocystidia and there are fibrous hyphae sparsely present in the subiculum which turn brown in Melzer's solution, but there are no basidia or spores. Because of this situation *C. rigescens* has been omitted from the synonomy. Should the name ever become of importance a type would need to be selected from one of the three different species represented in the Curtis herbarium under this name.

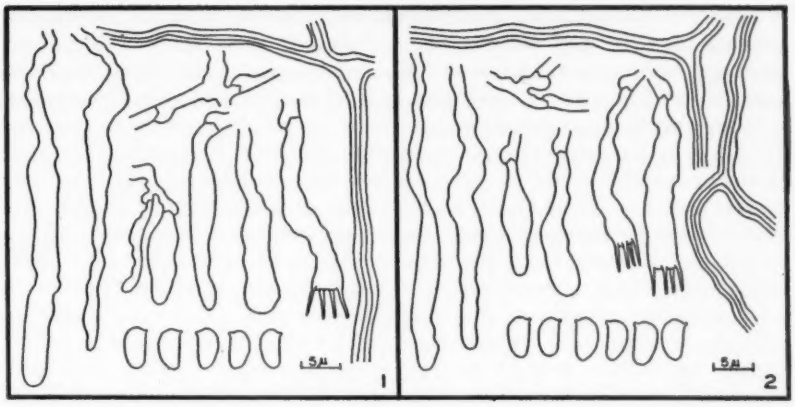
That *C. galactinum* occurs in Europe is evident from the four specimens cited above—two from Czechoslovakia and one each from Poland and Finland. In Europe, however, the species has commonly been referred to as *C. odoratum* var. *alni* (Fr.) Bres. This variety was established by Bresadola in 1920 and based on *Thelephora alni* Fr. and *Stereum alneum* Fr. Bresadola (6) also cites *Thelephora suaveolens* Fr. and *Stereum suaveolens* Fr. as synonyms of his variety. According to J. Eriksson, of the Institute of Taxonomic Botany at Uppsala (in litt.), no specimen of either *Stereum alneum* or *Stereum suaveolens* is now present in the Fries herbarium. From Bresadola's comments it is not possible to determine whether or not he had seen an authentic specimen. Until such a specimen is found and re-examined in the light of present knowledge there seems insufficient basis for reducing *C. galactinum* to synonomy.

There can be little doubt that *C. galactinum* will be found to occur in Sweden. Two collections made by L. Romell in the vicinity of Stockholm, determined by V. Litschauer as *Corticium odoratum* var. *alni*, have been examined. Both show clamps in the thin walled hyphae and the thick walled hyphae lack the characteristic branching of *C. odoratum*. Both specimens, however, are in poor condition and lack spores and we have hesitated to record these collections as certainly *C. galactinum*.

We have omitted also citation of three collections made by W. B. Cooke on Mt. Shasta, Calif., which had been identified as *C. galactinum*. These collections show much more branching of the thick walled hyphae than is typical of *C. galactinum* and in that respect are more like those in *C. odoratum* but the spores are small and comparable to those of *C. galactinum*. Assignment of these three Californian collections to either species is doubtful.

In this connection the specimen from Poland cited above is of special interest. This specimen is in the Burt herbarium at the Farlow, and is from the herbarium of Bresadola. The packet is marked in Burt's hand "=Corticium galactinum (Fr.) Burt", but was not cited under that species in his monograph of the genus (11). It is apparently one of the collections cited by Bresadola (5) in his earlier paper on Fungi Polonici. In that paper he listed the collections as on *Pinus*, *Alnus*, and *Populus* under *Stereum odoratum* Fr. but in his description he gives the small spore size, $4.5 - 6 \times 2 - 3\ \mu$. This is the spore size given in his description of *Stereum odoratum* var. *alni* (Fr.) Bres. in the 1920 paper. It seems probable that this collection from Poland was, in part, the basis of his concept of the variety.

It is evident that *C. odoratum* var. *alni* sensu Bresadola in Bourdot and Galzin is the same as *C. galactinum* since the description published by these authors corresponds closely with that of the latter fungus and examination of European specimens of *C. odoratum* var. *alni* reveals no significant difference from *C. galactinum*. The microscopic characters of these fungi agree in size and morphology. Camera lucida drawings of both Canadian and European collections of *C. galactinum* are presented for comparison in Figs. 1 and 2.



The microscopic structure of *Corticium galactinum.*

FIG. 1. *C. galactinum* – showing gloeocystidia, basidia, spores, thin-walled hyphae, and fibrous hyphae; drawn from the portion of the type specimen referred to in the text.

FIG. 2. *C. galactinum* (*C. odoratum* var. *alni*) – showing structures comparable to those in Fig. 1, but drawn from a Czechoslovakian specimen on *Abies alba* collected by Dr. A. Pilát.

All figures drawn to a uniform scale with the aid of a camera lucida from potassium hydroxide – phloxine mounts and reproduced at an approximate magnification of 1600×.

Corticium odoratum

Corticium odoratum (Fr.) B. and G. also resembles *C. galactinum* in most respects, possessing similar gloeocystidia and flexuous basidia. These two species, however, differ in three salient characteristics: the spores of *C. odoratum* are larger than those of *C. galactinum*, averaging about 8 × 5 μ; clamp connections, which are regularly present in the thin-walled hyphae and at the bases of the basidia of *C. galactinum* are lacking in *C. odoratum*; and the fibrous hyphae which are sparsely branched and threadlike in *C. galactinum* show a closer dendritic type of branching in *C. odoratum.*

Because of the similarity that exists between these two species it was deemed advisable to prepare the following description of *C. odoratum* for comparison with *C. galactinum:*

Corticium odoratum (Fr.) Bourdot and Galzin, Hyménomycètes de France, p. 225. 1928.

Thelephora odorata Fr. ex. For. Syst. Mycol. 1 : 445. 1821; Elenchus Fung. 1 : 207. 1828.

Stereum odoratum Fr. Epicr. Syst. Mycol. p. 553. 1838; Hym. eur. p. 644. 1874; Bresadola, Ann. Mycol. 1 : 92. 1903; Ann. Mycol. 18 : 63. 1920; nec *Stereum odoratum* sensu Karst., Bidr. Känned. Finl. Nat. Folk, 25 : 309. 1876.

Fructification white to cream-colored or ochraceous, even, adnate, coarsely pruinose, closely and indeterminately effused, thinning at the margin, perennial, varying in thickness to 500 μ or more, with age exhibiting a slight zonation when stained with phloxine, subiculum coriaceous, mostly composed of fibrous, nonseptate hyphae which are hyaline to slightly yellow in color and $1-3.5$ μ in diameter, closely compacted and with thick refractive walls becoming reddish-brown in Melzer's solution, frequently branching with several branches arising closely together, tips of fibrous hyphae at surface of fructification becoming narrowed, branching in an antlerlike manner, thin-walled hyphae from which the gloeocystidia and basidia arise are without clamp connections, gloeocystidia flexuous, elongate-cylindrical $40-100 \times 3-6$ μ, nearly colorless or with fragmented refractive contents, rather infrequent, interspersed in the subiculum and attaining the surface or projecting slightly beyond the level of the hymenium, hymenium weakly developed, basidia at first obpyriform, becoming long, cylindrical flexuous or ventricose-clavate $25-50 \times 3.5-5.5$ μ, emerging beyond the surface of the fructification to 5 μ, bearing four slender, upright sterigmata $4-5$ μ in length; basidiospores ellipsoid $6.5-9 \times 3.5-5$ μ, hyaline, flattened slightly on one side with prominent lateral apiculus, walls slightly thickened, smooth, non-amyloid.

Sporophores occurring on the underside of logs of pine and other conifers and hardwoods, Canada (Ontario) and Europe. Common in Northern Europe.

Specimens examined:

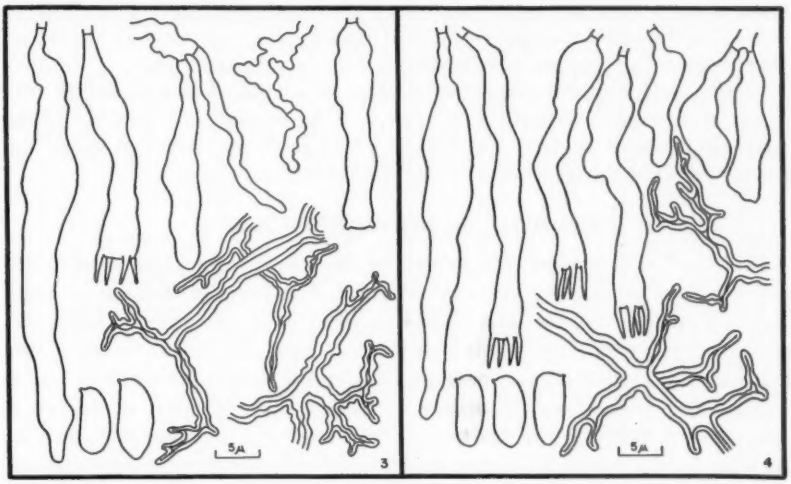
Ontario: on *Abies*, Cattle Island, Lake Timagami, T.F.R., H. S. Jackson, *23039*; on *Pinus*, Bear Island, Lake Timagami, T.F.R., Aug. 28, 1937, H.S.J., *12905*.

Czechoslovakia: on *Abies alba*, Carpatorossia, in valle rivi Liščenka prope vicum Trebušany, Aug. 1936, A. Pilát, PR; on *Picea excelsa*, Carpatorossia, in valle rivi Berlebaš prope vicum Trebušany, Aug. 1937, A. Pilát, PR.

Sweden: on rotten log of *Pinus* or *Abies*, Femsjö, L. Romell, Burt herb., FH; on *Pinus*, Femsjö, Aug. 27, 1899, L. Romell 213, Burt herb., FH; on *Quercus robur*, Småland, Femsjö parish, Björkeråknen, July 9, 1929, J. A. Nannfeldt *2313* UPS; on decaying coniferous board, Småland, Femsjö parish, Valsabo, Sept. 12, 1949, Seth Lundell *6147* and J. Stordal, UPS; on Nadelholts, Femsjö parish, Langbrolid, Sept. 6, 1899, L. Romell, ex herb. Burt, FH; on the underside of decaying, dry coniferous trunk, Småland, Femsjö parish, the W. side of Prästgårdsskogen, Aug. 13, 1937, Seth Lundell *1492* (Fungi Exs. Suecica *570*).

The gloeocystidia in *C. odoratum*, while certainly present, are not as evident as in *C. galactinum* and are much less abundant and easily overlooked. The contents tend to become granular and separate from the wall which can then be demonstrated only by most careful observation.

It should be noted that the two collections recorded from the Timagami Forest Reserve, Ontario, (TRT *12905, 23039*) correspond in all essential details with European specimens of *C. odoratum*. The spores appear to average slightly longer in the Canadian collections. The general conformity in morphological features, between Canadian and European specimens of *C. odoratum*, is demonstrated in the camera lucida drawings that appear in Figs. 3 and 4, respectively.



The microscopic structure of *Corticium odoratum*.

FIG. 3. *C. odoratum* – showing gloeocystidia, basidia, spores, and hyphae; drawn from a Canadian specimen (TRT *12905*).

FIG. 4. *C. odoratum* – showing features closely comparable to those shown in Fig. 3 but drawn from a European specimen ex Prague Herbarium.

All figures drawn to a uniform scale with the aid of a camera lucida from potassium hydroxide – phloxine mounts and reproduced at an approximate magnification of 1600×.

Formerly, in North America at least, the morphology of *C. galactinum* was considered to be distinctive and specimens have often been identified as this species without the confirmation afforded by spore measurements. Numerous nonsporulating collections have been ascribed to *C. galactinum* and included in herbaria on the basis of general morphology alone. The discovery of *C. odoratum* in Ontario not only makes this practice untenable but also suggests the possibility that some of these specimens may have been wrongly labelled. The nature of the fibrous hyphae and the presence or absence of clamps, as noted above, appear to furnish positive criteria for separation of the two species when spores are lacking.

Relationship

Recently, Jackson (16) has pointed out that a group of species included in *Corticium* are characterized by a coriaceous subiculum made up largely of narrow, fibrous hyphae that turn a characteristic reddish-brown in Melzer's solution, and possess other characters that suggest close interrelationships. In this related group *C. galactinum* is listed together with *C. odoratum*, *C. portentosum* B. and C., *C. abeuns* Burt (=*Gloeocystidium ochreoleucum* Bres. and Torr.) and *C. praestans* Jackson. It is suggested that although these may be retained conveniently for the present in Section 10, Trichostroma, of the useful Bourdot and Galzin classification, they all show characters that suggest a possible relationship with the genus *Vararia* (=*Asterostromella*) and may ultimately find a place as a natural section within that genus or as a separate but related genus. The manner of branching of the basal thick walled hyphae of *C. odoratum* is strongly suggestive of the dichophyses found in the subiculum of species of *Vararia*.

Cultural and Interfertility Studies

Fruiting bodies of *Corticium galactinum* (Fr.) Burt from which single spore cultures were obtained were collected during the summer and fall of 1948. Revived specimens were later waxed to the top of moist Petri dishes till spores were shot onto the sterile agar below. Spores were removed from this deposit with a dry platinum needle and three dilutions were made in sterile water. Ten drops of each dilution were spread over the surface of very clear agar in Petri dishes. The dishes were then incubated at room temperature. As soon as the spores had germinated sufficiently for the sporelings to be clearly distinguishable under the high power binocular, single germinating spores were isolated from the most suitable dilution plate.

Spore isolation was carried out using a flattened spear-pointed platinum wire. A small block of agar was cut out with the spear-point and this transferred with the sporeling to an agar slant. Care was exercised to avoid polysporous cultures though one or two obvious polysporous cultures were isolated at the same time as each set of monosporous cultures.

Monosporous cultures were paired in all possible combinations in the interfertility experiments described hereafter. Cultures, the genetical constitution of which was demonstrated by interfertility studies, were used to test the interfertility between fruiting bodies from different sources. The fruiting bodies used in these experiments and as the basis for a new description of the species have been deposited in the mycological herbarium of the Department of Botany, University of Toronto (TRT *23337*, *23338*).

The experiments of Kniep (17, 18) with *Schizophyllum commune* Fr. demonstrated the occurrence of tetrapolar heterothallism among the Hymenomycetes. Subsequent work by Brunswick (8) with *Coprinus fimentarius* (L.) Fr., Hanna (15) with *Coprinus lagopus* Fr., and Vandendries (33) with

Coprinus micaceus (Bull.) Fr. confirmed the presence of multiple allelomorphs at the loci responsible for heterothallism. Mounce (23, 24, 25), Nobles (26, 27), Macrae (20, 21), Biggs (2, 3), *et altera*, continued to focus attention upon the study of heterothallism. The results and implications of these

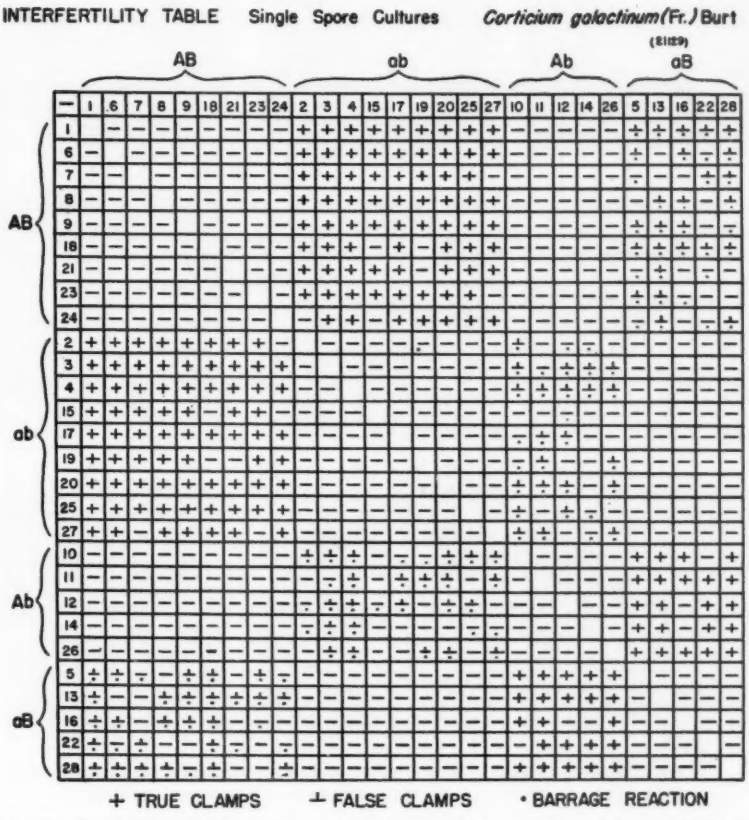


FIG. 5. Interfertility table showing the results of pairing, in all possible combinations, a series of 28 monosporous cultures from a single fruit body of collection number *21129* (TRT *23337*), *C. galactinum*, Wilno, Ontario, July 24, 1948.

studies have been examined by Whitehouse (37) in his critical review of data published to date. It is now generally accepted that fruit bodies of heterothallic species of Hymenomycetes normally have two allelomorphs at each locus for heterothallism, and arise from a single cell containing a pair of nuclei of different mating types. All the evidence suggests that both bipolar species (with one locus) and tetrapolar species (with two loci) may possess multiple allelomorphs at the loci responsible for fertility.

Two interesting phenomena have been described in tetrapolar species. Vandendries and Brodie (34) and Brodie (7) studied a phenomenon, which they named the "barrage reaction", in the tetrapolar species, *Lenzites betulina* (L.) Fr. These workers observed that when two haploid mycelia are grown

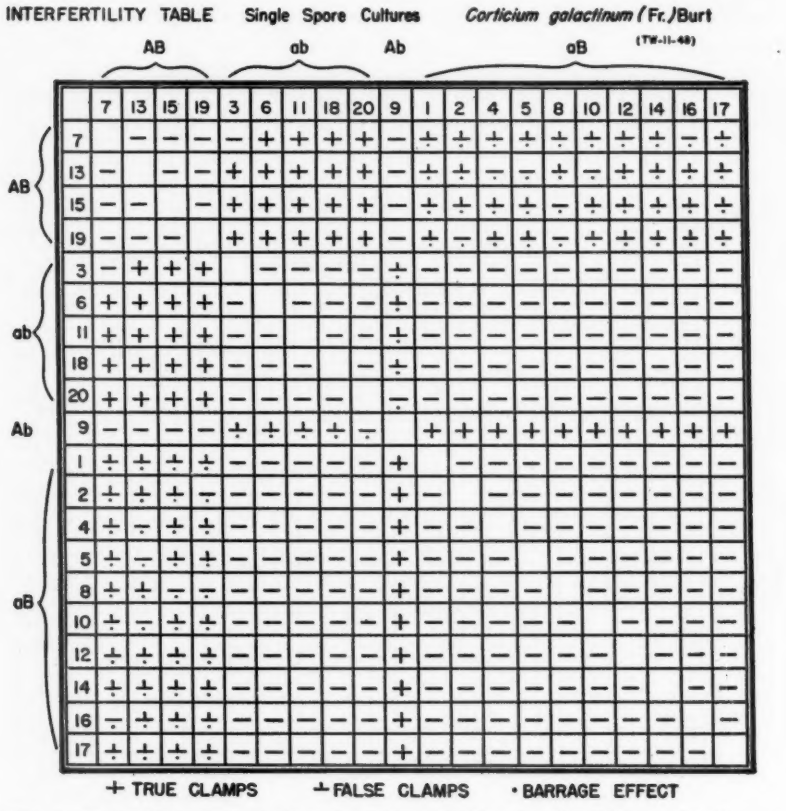
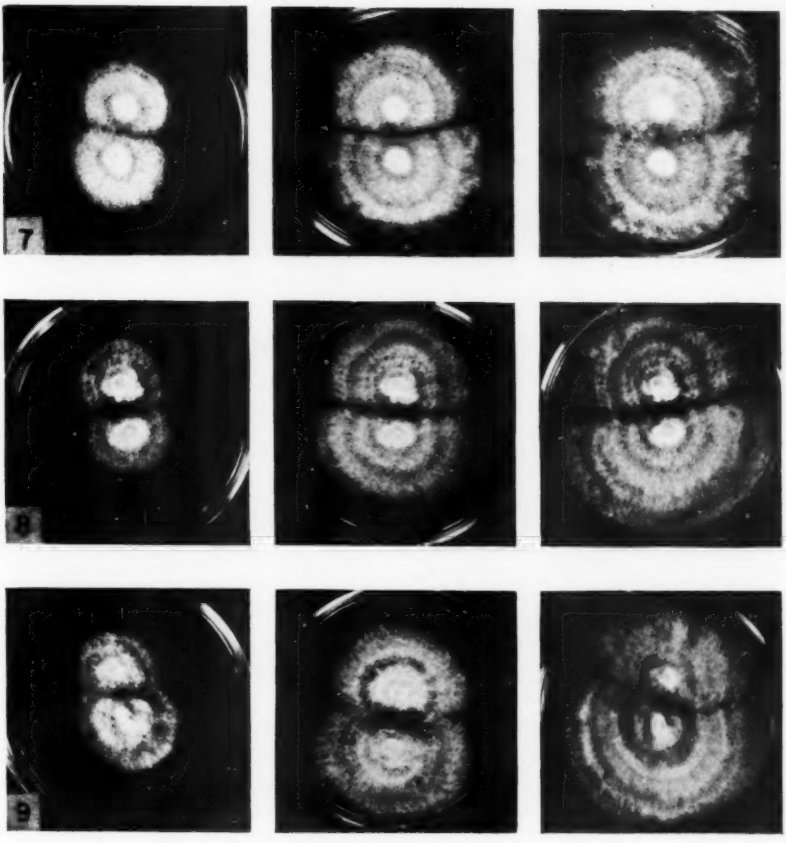


FIG. 6. Interfertility table showing the results of pairing, in all possible combinations, a series of 20 monosporous cultures from a single fruit body of collection number TW-11-48 (TRT *23338*), *C. galactinum*, Timagami, T.F.R., Ontario, Sept. 1, 1948.

side by side on nutrient agar they tend to intermingle freely or to exhibit a mutual aversion, the "barrage effect" in which the separating zone is bridged by only a few hyphae or none at all. They state that in *Lenzites betulina* the presence of a "barrage" is correlated with the genetic constitution of the mycelia concerned. A "barrage" appears when one pair of fertility factors is present in heterozygous condition, but its manifestation is completely independent of the homozygosity or heterozygosity of the other pair.

PLATE I



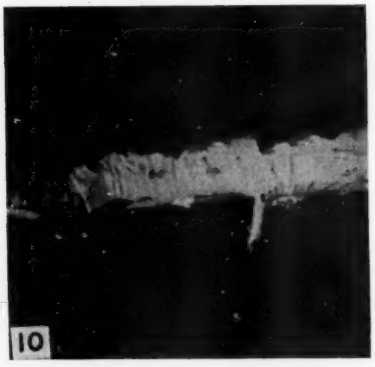
Corticium galactinum – monosporous cultures from collection in No. TW-11-48, on malt agar representing:

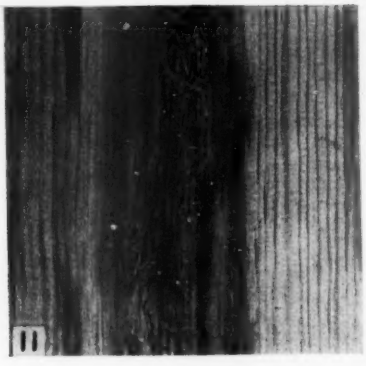
FIG. 7. The fertile combination of Cultures 11 and 13 ($AB \times ab$);

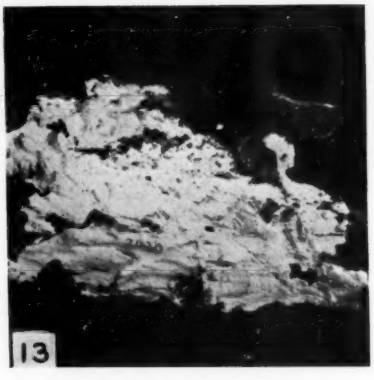
FIG. 8. The nonfertile combination of Cultures 10 and 19 ($AB \times aB$);

FIG. 9. The nonfertile combination of Cultures 9 and 15 ($AB \times Ab$); photographed after 9, 15, and 21 days.

It should be noted that in Fig. 7 the temporary "barrage" which is clearly evident at 15 days disappears with the bridging of the gap, the formation of clamp connections, and mutual diploidization. Diploid hyphae may be seen in the area separating the two monosporous mycelia at 21 days. In Fig. 8 the "barrage" persists throughout, is bridged rarely, and false clamp connections are formed when "illegitimate fusions" occur. In Fig. 9 the nonfertile combination is characterized by an intermingling of hyphae without fusions or the formation of a "barrage".







Stringy yellow butt rot caused by *Corticium galactinum*.

FIG. 10. Fruiting body of *C. galactinum* on root of white pine.

FIG. 11. Butt log of white pine showing center of log completely destroyed by this fungus.

FIG. 12. Longitudinal section through a rotted log illustrating the stringy nature of this butt and root rot.

FIG. 13. Fruiting body of *C. galactinum* formed on bark on the underside of a white pine log lying on the ground.

Biggs (3), on examining the pairing reactions of 25 monosporous mycelia of a single fruit body of *Cytidia salicina* (Fr.) Burt, observed that both the "barrage" phenomenon and the formation of false clamp connections occurred when the paired mycelia possessed one member of an allelomorphic pair in common, arbitrarily designated by her as either (BB) or (bb). She stated that, in this species, the formation of a "barrage" and the formation of false clamp connections were evidently controlled by the same pair of fertility factors.

Figs. 5 and 6 present, in tabular form, the results that were obtained when monosporous cultures from single fruit bodies of *Corticium galactinum* were paired in all possible combinations. Examination of these tables reveals that this species exhibits a tetrapolar heterothallism. It is evident that a single fruit body of *C. galactinum* will produce four kinds of basidiospores that give rise to haploid mycelia of differing diploidizing potentialities. Since interfertility appears to be controlled by two pairs of allelomorphs, basidiospores and resultant mycelia may be conveniently represented as (*AB*), (*ab*), (*Ab*), and (*aB*). When taken two at a time, these mycelia may be combined in six different ways. Mutual diploidization is effected between the two mycelia of the combinations, (*AB*) × (*ab*) and (*Ab*) × (*aB*), in which there are no factors in common; but not between the two mycelia of the combinations, (*AB*) × (*aB*), (*AB*) × (*Ab*), (*ab*) × (*aB*), or (*ab*) × (*Ab*), in which there is one factor in common.

A "barrage reaction" occurs in the nonfertile combinations (*AB*) × (*aB*) and (*Ab*) × (*ab*). The occurrence of this phenomenon in *C. galactinum* is best explained when one accepts the theory advanced by Vandendries and Brodie. It has been observed that an aversion also appears in the fertile combinations (*AB*) × (*ab*) and (*Ab*) × (*aB*), although in these cases the "barrage" is transient and disappears as the hyphae move across the original gap to fuse and diploidize each other. When "barrages" occur in nonfertile combinations, however, the gap is bridged rarely or not at all; and those "illegitimate fusions" that occur result in the formation of false clamp connections. In both these instances a "barrage" has developed where the (*A*) (*a*) factorial pair is present in a heterozygous condition regardless of the heterozygosity or the homozygosity of the (*B*) (*b*) factorial pair. In short, a "barrage" may be formed in the following factorial combinations: (*AB*) (*ab*), (*Ab*) (*aB*), (*AB*) (*aB*), and (*Ab*) (*ab*). The occurrence of the "barrage reaction", transient or permanent, would seem to be the result of a heterozygous condition in one of the fertility factors, here arbitrarily represented as the (*A*) (*a*) allelomorphs.

The appearance of false clamp connections, on the other hand, seems to be controlled by the assortment of both factorial pairs. False clamp connections were found only when the (*B*) (*b*) allelomorphs occurred in a homozygous state, or as Biggs (3) opined: in a community of the (*B*) or (*b*) factors. However, the manifestation of clamp connections did not appear to be determined

entirely by the state of the (*B*) (*b*) factorial pair; false clamp connections only occurring when the (*A*) (*a*) allelomorphs were present in the heterozygous condition necessary in fertile combinations. No false clamp connections were formed in those nonfertile combinations that resulted when mycelia of the same genetic constitution were paired, viz.: (*AB*) × (*AB*) or (*ab*) × (*ab*). False clamp connections did appear when one pair of factors, here designated as (*A*) (*a*), were present in heterozygous condition, and the second pair, here represented as (*B*) (*b*) were present in a homozygous condition.

In the remaining two nonfertile combinations, (*AB*) × (*Ab*) and (*ab*) × (*aB*), hyphae of the paired monosporous cultures intermingled without the expression of an aversion or false clamp connections.

The appearance of the above-described combinations of monosporous hyphae growing on nutrient agar has been pictured in Figs. 7, 8, and 9.

The interfertility between monosporous cultures obtained from two fruit bodies of *C. galactinum* collected in different areas is depicted in Fig. 14. The mating types of monsporous mycelia from each of these two fruit bodies were

INTERFERTILITY TABLE *Corticium galactinum* (Fr.) Burt

Single Spore Cultures From Two Sporophores

FRUIT BODY Tw-II-48

FRUIT BODY 21129		AB	ab	Ab	aB
		13	11	9	4
A_1B_1	8	+	+	+	+
a_1b_1	3	+	+	+	+
A_1b_1	12	+	+	+	+
a_1B_1	28	+	+	+	+

\+ True Clamps Formed

FIG. 14. Interfertility table showing the results of pairing, in all possible combinations, the four mating types of monosporous mycelia from two different collections of *C. galactinum*.

established in the individual interfertility tests described above. Each of the four mating types of mycelia from one fruit body was paired in all possible combinations with the four similarly constituted mycelia from the second fruit body. In all cases these pairings produced clamp connections, indicating

the presence of multiple allelomorphs for fertility in these specimens of *C. galactinum*. Since no basidia occurred in culture it was not possible to test the interfertility in the F_1 generation.

Acknowledgments

The writer wishes to express his indebtedness to Dr. H. S. Jackson, Department of Botany, University of Toronto, for advice and stimulating criticism, and for supplying much of the materials used in the taxonomic portion of the study; to Dr. J. H. Craigie, Dominion Botanist, for permission to use certain data in postgraduate studies, to Dr. M. K. Nobles for assistance in cultural identifications of wood-rotting fungi, to Miss Jean Robinson for assistance with photographs and enlargements; and to Mr. Z. Patrick and Miss E. R. Dearden for aid in the preparation of the manuscript. Other acknowledgments are made in the text.

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NOTE

A Note on the Antibiotic Activity of 40 Species of Wood-rotting Fungi[1]

During a carbon-balance survey of surface cultures of 40 species of wood-rotting fungi (1), the culture filtrates of each species were tested for antibiotic activity against *Staphylococcus aureus* (Oxford H strain), *Escherichia coli*, and *Mycobacterium phlei*. The culture filtrate was sterilized by filtration through a Morton filter and diluted to concentrations of 1 : 10, 1 : 20, and 1 : 40 with broth of the following composition: Bacto beef extract – 1.5 gm., Bacto yeast extract – 1.5 gm., Bacto peptone – 5.0 gm., glucose – 10 gm., water – to one liter. Each concentration was inoculated with a 24 hr. broth culture of the organisms listed, with the usual positive and negative controls. After 48 hr. incubation at 37° C., the tubes were examined for the presence or absence of growth.

Under optimal conditions for the test organisms, none of the culture filtrates tested had appreciable antibiotic activity. In broth dilutions of suboptimal pH, the culture filtrate of *Poria xantha* A74 inhibited the growth of a number of mycobacteria but in broth buffered at pH 7.0, the inhibitory dilution was below 1 : 10. It had no activity against *Mycobacterium tuberculosis* in the oleic acid – serum albumin medium of Dubos.

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* *Biochemist, Industrial Utilizations Investigations.*

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